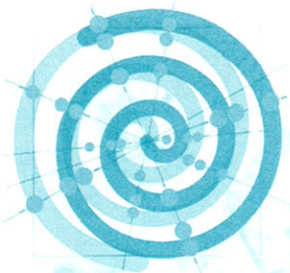
Research Institute of Molecular Pathology

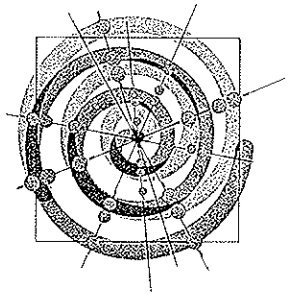
V I E N N A B I O C E N T E R

IMP
1995



Cover: Increased apoptosis and reduced vascularization in ß cell tumors of transgenic mice that have been treated with anti-angiogenic compounds. Front-cover: placebo-treated; back-cover: treated. For details see report by group Christofori. (Foto: Gerhard Christofori/Hannes Tkadletz, I.M.P.)

Scientific Report
1995



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The I.M.P. conducts basic research within the International Boehringer Ingelheim Group

Contents

Impressum:

Published by:	I.M.P., Dr. Bohr-Gasse 7, A-1030 Vienna, tel. (+43 1) 797 30
Managing Director:	Prof. Dr. Max L. Birnstiel
Administrative Director:	Dr. Nikolaus Zacherl
Editor:	Heidemarie Hurtl
Layout:	Hannes Tkadletz
Printed by:	Agens-Werk Geyer + Reisser, 1050 Vienna

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Introduction

The Research Institute of Molecular Pathology is a research organization financed nearly exclusively by Boehringer Ingelheim. The IMP, as a center of excellence, attempts to offer its scientists the best possible working conditions. Our institute is based on the premise that turnover of scientists is of paramount importance allowing to make a fresh start each time a new collaborator joins one of the research groups. Thus, Ph.D. students are allowed a maximum of 4 years, but most of them finish their thesis by 3 1/2 years. After their final exams, most students travel to the USA for postdoctoral education and experience. New Ph.D. candidates are chosen exclusively from the yearly intake of students of the international Ph.D. program which the IMP initiated a few years ago and which is now run jointly with the University departments of the Vienna Biocenter.

Postdocs have a time limit of 6 years, but in practice most of them leave after 3 years unless they are promoted to staff scientists which allows them to stay on for an additional 3 + 2 years. Senior Scientists are on a rolling tenure of three years. Group leaders stay 5 + 3 years and then are replaced by new team leaders. In this vein, Lisa Ballou left the IMP to follow a call as Assistant Professor of Pharmacology at the University of Texas in San Antonio and Martin Zenke became Associate Professor at the Max Delbrück Center in Berlin-Buch. We wish them both much success and satisfaction in their new endeavors. Lukas Huber coming from the University of Geneva will join the IMP on 1.1.96 and Jan-Michael Peters from Harvard later in the year.

Members of the IMP were busy organizing conferences and workshops throughout the year. The traditional IMP Spring Conference, the 6th in a series, was organized by Erwin Wagner and had as a theme: Interfaces between Cancer and Development (see page 123). In June, Max L. Birnstiel organized an ESO (European School of Oncology) Workshop on Gene Therapy of Cancer in Europe and Andreas Weith the 2nd International Workshop on Human Chromosome 1 Mapping in September. Finally, Thomas Jenuwein of the IMP and Manuela Baccarini from the University organized a joint Vienna Biocenter recess.

In the course of an evaluation of the biochemistry labs in Austria, we were visited and interviewed by an international panel which gave us the highest praise. Thomas Böhm et al. from Kim Nasmyth's group received a research prize from Hoechst and Kim Nasmyth received the FEBS silver medal and was appointed Honorary Full Professor at the University of Vienna.

Throughout 1995 we watched closely the phase I human trial on tumor vaccines for malignant melanomas being carried out by Prof. Georg Stingl at the Department of Dermatology of the General Hospital in Vienna (see chapter on Tumor Vaccines).

This year we had the great pleasure of dedicating our library, run jointly by the IMP and the University departments of the Biocenter, to the Vienna-born Nobel Prize winner Max Perutz who honored us by attending the opening ceremony and giving us a talk on his recent research.

Max L. Birnstiel
Managing Director

Tumor vaccines

Managing director	Max L. BIRNSTIEL
Staff scientist	Walter SCHMIDT
Postdoc	Gerhard MAASS (until Feb. 1995)
Postdoc	Tamás SCHWEIGHOFFER
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Laboratory technician	Helen KIRLAPPOS

Introduction

An invidious feature of neoplastic disease is that in many cases cancer cells are disseminated throughout the body and form occult micrometastases long before the primary tumor is discovered. Whereas the primary tumor in most cases is accessible to surgical removal, attempts to cure malignancies must be directed at eliminating the occult metastases which otherwise flare up and kill the patient at a later stage of the disease. Our team, like many other groups internationally, have concentrated initially on the generation of whole cell tumor vaccines expressing IL-2 in mouse models as a stepping stone for later clinical investigations. In this work, our IMP transfection method AVET (**a**denovirus **e**nhanced receptor mediated **t**ransferrinfection) proved to be of especial utility, since vaccines expressing high levels of IL-2 could be prepared (Schmidt *et al.*, 1995a) without the recourse to cell cloning.

Our efforts in the field of tumor vaccines culminated this year with the accruement of information from a phase I trial with terminally ill patients in the Department of Dermatology, Division of Allergic and Infectious Diseases of Prof. Georg Stingl at the General Hospital in Vienna (Austria) and in the Dermatology Department of Prof. E. B. Bröcker in Würzburg (Germany). These clinical studies were made possible through the support of Dr. Ernst Wagner and his team at Bender in Vienna where the human vaccines were prepared. The trial showed that the vaccine was well tolerated by patients and many interesting anecdotal data were already generated which suggested we are on the right track with the IL-2 secreting vaccines prepared by the IMP transfection procedure. The trial is expected to be completed in Spring of 1996 and definite conclusion will have to await the end of this trial. Meanwhile, our group has continued to study the molecular and cellular mechanisms which lead to tumor immunity when tumor cells are transfected with cytokine expression vectors, irradiated and applied as whole cell vaccines and has developed alternative vaccination strategies.

Work carried out in collaboration with Prof. Georg Stingl, Department of Dermatology, Division of Allergic and Infectious Diseases, showed for mouse models that anti-tumor immunity after vaccination with IL-2 secreting, irradiated vaccine is dependent on both $CD4^+$ and $CD8^+$ T cells (Zatloukal *et al.*, 1995). Based on these findings and an extensive immunohistological investigation of the site of vaccination we proposed a three stage model (Maass *et al.*, 1995) to explain the generation of protective immunity: (a) tumor antigen uptake and processing at the site of injection by antigen presenting cells, (b) migration of antigen presenting cells into the regional draining lymph nodes where T cell priming occurs and (c) recirculation of activated cytotoxic T cells that recognize distant tumor deposits and initiate their elimination. We have provided further proof for this hypothesis by analyzing the various single steps of this chain of events.

Possible involvement of macrophages as antigen presenting cells in transferring the tumor antigen to the peripheral lymph nodes

Michael Buschle and Manfred Berger

Two different models have been postulated for the priming of tumor specific T lymphocytes: a) tumor cells directly prime naive T cells and b) the three stage model where naive T cells are activated in secondary lymphoid organs by professional antigen presenting cells which have taken up tumor antigens in the periphery.

There are several theoretical as well as practical arguments against the direct priming model including that in our system T cells were never found at

the site of immunization, rather a prominent infiltrate of F4/80+ macrophage like cells was present. In addition, signs of T cell activation were only detected in secondary lymphoid organs (Maass *et al.*, 1995). Furthermore, this model conflicts with T cell migration patterns (see below).

In order to further strengthen our hypothesis we attempted to reconstruct the initial steps of the three stage model, namely tumor antigen uptake and processing by antigen presenting cells (APC) in vitro. Subsequently, tumor antigen loaded APCs were injected into syngeneic animals and subjected to a lethal challenge of live tumor cells with a number corresponding to 20-50 times the tumorigenic dose.

APCs were generated from bone marrow cultures treated with M-CSF-1 or GM-CSF following established protocols. After 7-9 days in culture cells were treated with a freeze thaw lysate of tumor cells -the "tumor antigen"- and incubated for a further 24 hours. During the loading process APCs were treated with IFN-γ in order to enhance MHC class II expression and to promote antigen processing. Day 7-9 cultures were positive for the markers F4/80, Mac-1 and other molecules typically expressed on bone-marrow derived macrophage-like APCs. The cultures showed strong phagocytic activity.

As illustrated in **Fig. 1** partial protection from tumor challenge is indeed achieved following treatment with antigen-loaded APCs but not in controls, further validating our hypothesis of indirect priming of naive T cells by APCs. These findings may have several implications for improved vaccine design: a) since APCs present antigens, MHC non-matched allogeneic vaccines may be equally effective as autologous preparations. b) the role of MHC class II antigens needs to be evaluated. c) assays for vaccine efficiency may be developed based on APC

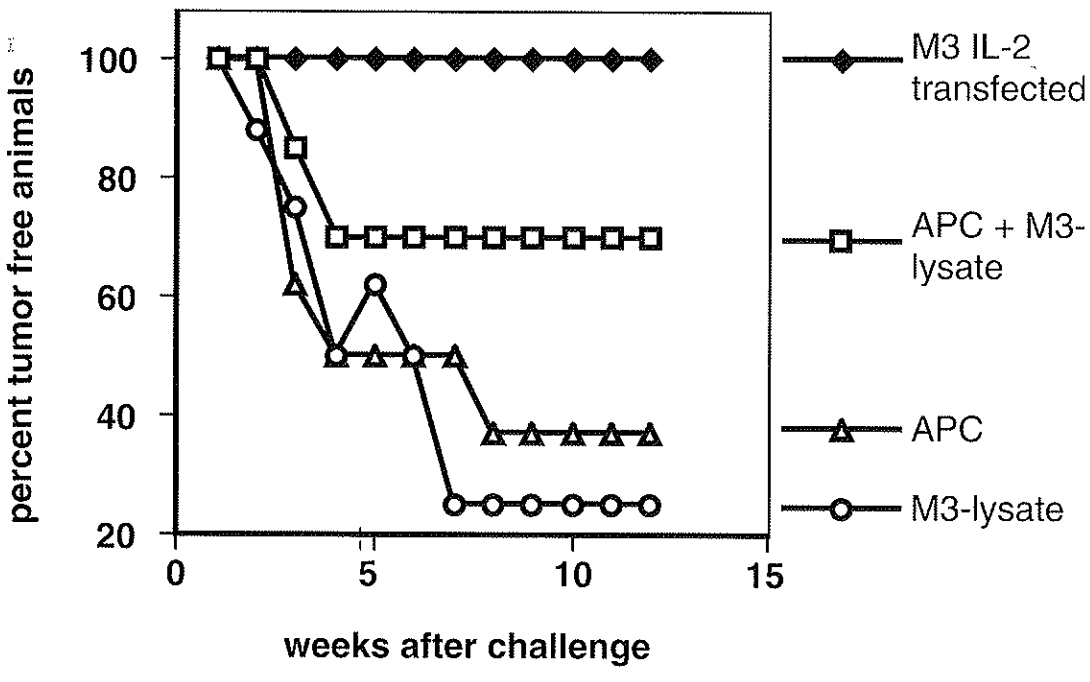

Fig. 1: Induction of anti-tumor immune responses following vaccination with tumor cell lysate loaded antigen presenting cells (APC). DBA/2 mice were vaccinated twice with APCs loaded with M3 tumor cell lysate ( ), APCs alone ( ), or M3 freeze-thaw lysate alone ( ) followed by a challenge with $1x10^5$ parental M3 cells. A standard cytokine transfected vaccine (M3 transfected with IL-2) served as reference.

recruitment and activation. d) next generation cancer vaccines, e.g. using tumor antigen derived peptides, should aim at optimal induction of APCs in order to achieve effective vaccination.

In direct comparison with an optimized cytokine transfected vaccine antigen loaded APCs were consistently less effective (**Fig. 1**). Several reasons may be responsible for this phenomenon. One possibility would be that the APC generation, activation and loading protocols applied may not be optimal. Alternatively, cell types other than classical APCs such as granulocytes may in addition be required for optimal vaccination efficiency. These points are addressed in ongoing studies.

Depletion of naive T cells of the peripheral lymph nodes abrogated systemic anti-tumor protection conferred by IL-2 secreting vaccines

Tamás Schweighoffer

Next we wanted to provide evidence that the immune rejection of tumor deposits depends on activation of naive T cells (rather than memory cells) and occurs distinct from the vaccination site, downstream in the draining lymph nodes.

A fundamental difference exists between naive and memory subsets of T cells: it is the (activated) memory cells that interact with the vascular endothelium to enter tissues, while naive cells home preferentially to secondary lymphoid organs, such as peripheral lymph nodes. This migration is initiated by the interaction between L-selectin, expressed on naive T cells and the sulphated mucin GlyCAM-1 on the high endothelial venules (HEV) of the lymph nodes. It is known from experiments of others that naive T cells that have their L-selectin masked by the specific antibody MEL-14 are incapable of entering peripheral lymph nodes. Instead they are diverted into the spleen, leaving the peripheral lymph nodes virtually depleted of them.

First we established that naive T cells could be quantitatively removed from peripheral lymph nodes by the treatment of mice with MEL-14 antibody. Single cell suspensions of naive or MEL-14 depleted lymph nodes were incubated with an excess amount of unlabeled MEL-14 mAb (of rat origin) and stained with FITC-conjugated polyclonal anti-rat antibodies. As shown in **Fig. 2**, FACS analysis clearly showed that lymph nodes from naive mice exhibited strong fluorescence whereas lymph nodes from mice depleted by MEL-14 lacked this specific signal.

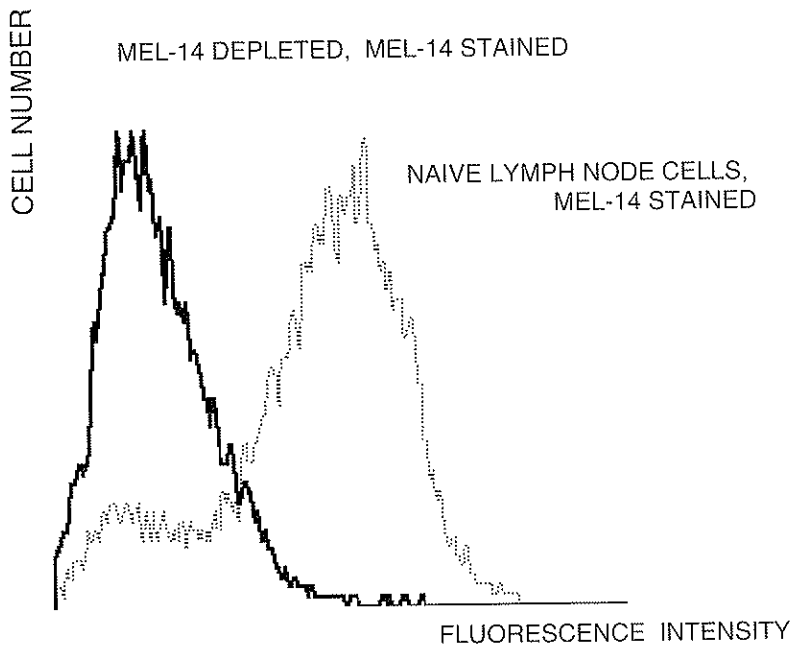

Fig. 2: FACS analysis of lymph node cells before and after depletion *in vivo* of mice with MEL-14 antibodies.

As shown in the key experiment of **Fig. 3**, pre-treatment of mice with MEL-14 antibodies at double the dose required to deplete L-selectin positive lymphocyte subsets prior to vaccination with IL-2 secreting vaccine completely abrogates the generation of anti-tumor immunity, stressing the importance of naive T cells (rather than memory cells) in this process and the peripheral lymph nodes as an environment for the generation of anti-tumor immunity. These findings strongly support our hypothesis of an activation of naive T cells in the draining lymph node (Maass *et al.*, 1995).

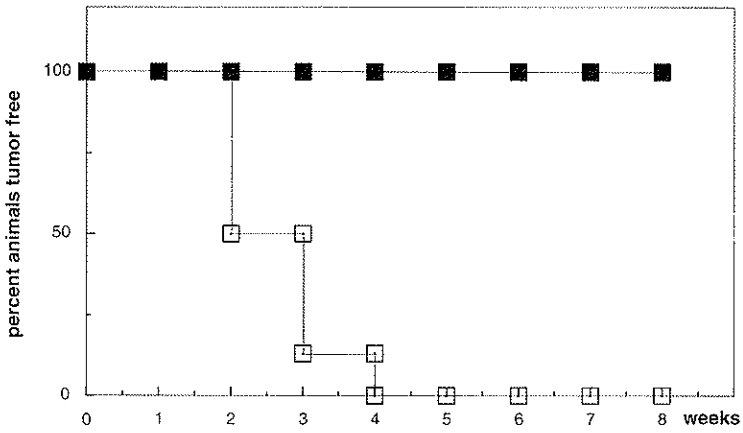

Fig. 3: Anti-tumor immunity generated in naive mice (■); MEL-14 depleted mice (□).

Transfection into M-3 mouse melanoma of a prototypic antigen that is a target of pre-existing memory response results in the development of anti-tumor immunity

Tamás Schweighoffer

As a result of host-tumor coexistence in cancer patients, only a limited host-antitumor response is generated as demonstrated by the successful isolation of tumor reactive cytotoxic T lymphocytes from the peripheral blood of cancer patients. However, these cells do not appear to be fully competent since tumors are not eradicated. This incompetence can be attributed to tolerance or silencing of reactive T cell populations, or, at the molecular level, to altered T cell receptor signaling transduction pathways. One way to break tolerance in experimental models is to generate *de novo* cytotoxic cells against tolerated antigen by specific immunization. Induction of anti-tumor immunity in tumor-tolerant hosts might be brought about by evoking an existing, well defined memory-type cellular response and redirecting it against tumor cells. This would require the modification of tumor cells to express the particular target of the memory response in addition to intrinsic tumor antigens. The pre-existing, specific memory T cells would access the immunogen, resulting in an efficient recognition and enhanced presentation not only of the genetically introduced antigen but also of tumor antigens residing on the tumor cell. Such a spreading of primary response to cryptic epitopes has been demonstrated by others at the molecular level. We anticipate that refocusing of the memory response should not only be able to break host tolerance, but may also result in a faster and more predictable build-up of anti-tumor immunity.

Immunization with Mycobacterium bovis BCG leaves behind a long lasting, in many cases life long immunity. In immune humans this protection can be characterized as a delayed-type hypersensitivity reaction that is generated primarily by Th-1 inflammatory T cells. In addition, a humoral response is also generated.

In order to demonstrate the feasibility of the above described approach, a hybrid protein based on the hsp65 protein of Mycobacterium bovis BCG (termed Heat 1) was used as the targeting antigen. This hybrid protein was constructed by fusing the signal- and GPI-exchange coding sequences of the mouse heat stable antigen (HSA) to the immunodominant portion of the BCG-derived hsp65. The gene encoding this peptide was transferred into the murine M-3 melanoma cells to generate a tumor vaccine. Mice, preimmunized with whole BCG or with purified recombinant protein, were vaccinated with Heat-1 expressing, irradiated M-3 cells and were subsequently challenged with a tumorigenic dose of parental-type M-3 cells.

For initial characterization, the Heat-1 hybrid protein was first expressed in COS-7 cells, since these cells permit high levels of expression through episomal replication of the chosen vector. The GPI-exchange motif at the N-terminal of the construct resulted in a final product that was directed to the outer surface of the cell membrane via a GPI-anchor, thus allowing identification of positive cells by FACS analysis. A panel of cross reactive mAbs that recognize members of the 65 kDa heat-shock protein family of related Mycobacteria was tested for detection of purified recombinant bacterial hsp65 and Heat-1 protein.

Expression of Heat1 was also successful in the mouse melanoma line M-3. A somewhat lower proportion of M-3 cells were high expressors as

compared to COS cells after AVET (generally 19-45% vs. 45-70%) ; this is likely due to the better transfectability of COS cells. Non-transfected COS-7 and M-3 cells do not react with any of the cross-reactive anti-hsp65 antibodies.

Mice were preimmunized with live BCG and rested 6 weeks before receiving the M3/Heat-1 immunizations. Upon challenge with a 100-fold tumorigenic dose of live M3 cells, only 1/8 developed a tumor, while all others remained tumor-free. In the non-vaccinated control group, 8/8 mice succumbed to tumor growth.

As a control, mice were immunized with whole, irradiated *E. coli* bacteria of the strain M1456 which harbors the plasmid for the recombinant hsp65 protein, but does not express it. As anticipated, the majority of these mice (6/8) developed tumors upon challenge with the parental-type M3 cells.

We also investigated the site of vaccination by immunohistology as in previous studies. In live BCG pre-immunized mice, macrophages constituted the overwhelming majority of infiltrating cells, as was previously the case of IL-2 secreting vaccines (Maass *et al.*, 1995), but granulocytes were not present. In contrast to IL-2 secreting vaccines, but in keeping with our expectations, a low, but significant, number of cells positive for anti-CD3 and anti-CD4 antibodies could be detected. Interestingly, large multigranular cells appeared at the rim of the inoculum, occasionally in close proximity to $CD4^+$ cells, which, based on their morphology, are most likely tissue mast cells. Mast cells are a rich source of a wide variety of cytokines and mediators, such as TNFα, IL-1, IL-4 and GM-CSF, which may promote the vaccination site reaction.

Our results indicate that immune memory can be redirected and utilized to target tumor cells. With most of the human population vaccinated against various pathogens (about 40 % with BCG) there is hope that such a strategy would be widely applicable for human cancer therapy.

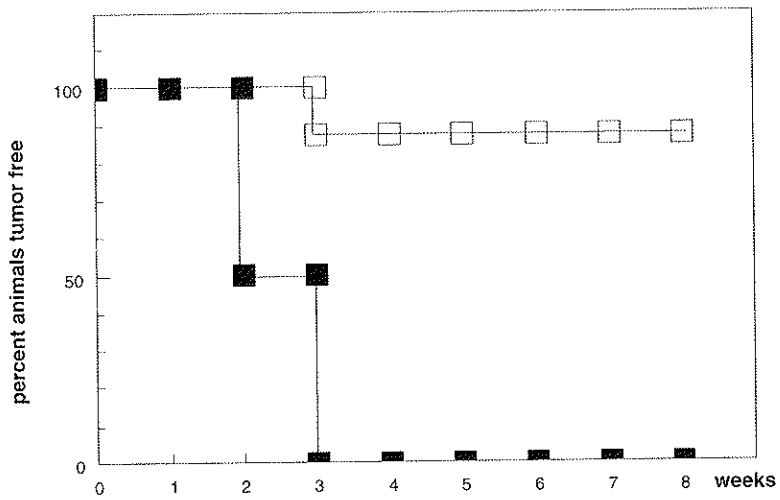

Fig. 4: Generation of anti-tumor immunity in naive (—■—) and BCG prevaccinated mice (—□—).

Transloading of immunogenic peptides, another new way to elicit strong tumor immunity

Walter Schmidt

Although the expression of immunomodulatory molecules such as IL-2 generates tumor vaccines which are increasingly shown to be efficacious also in humans, this method requires genetic modification of autologous tumor cells and therefore is patient specific. As a consequence, this strategy is extremely cumbersome and expensive. Even if these drawbacks did not exist it has to be noted that in most instances it will not be possible to generate IL-2 secreting tumor vaccines from (small) primary tumors because too few cells are recovered. Furthermore, experience has told us that only in about 2/3 of the cases vaccines can be provided, due to failure of culturing or efficient genetic modification with the IL-2 gene. This means that a sizeable proportion of patients will never profit from these new approaches. All our results show that tumor antigen presentation is effected by macrophages or macrophage-like cells

(see above and Maass *et al.*, 1995). Therefore, there is no need for a match between the MHC-type of the tumor vaccine with that of the host. This means that an alternative, technically simple, non-patient specific vaccination strategy is to use allogeneic tumor cells which can be prepared in bulk and stored until use.

In recent years, a whole series of tumor antigen peptides have been identified in both murine and human tumors. In theory it should be possible to use these peptides to elicit anti-tumor immunity. Since cellular rather than humoral immunity will have to be generated to combat distant metastases, ways have to be found to deliver tumor antigen peptides so that anti-tumor T cell populations are generated. The strategy described below represents a first step towards working out methods to elicit tumor immunity in a patient-nonspecific way.

Being of self origin, tumor cells are poorly or non-immunogenic and thus are often ignored by the immune system. However, in animal models, anti-tumor immunity has been shown by others to be very effective in the rejection of malignant cells in the context of minimal residual disease as soon as expression of a foreign gene is elicited, e.g. after viral infection or after transfection with an influenza haemagglutinin HA2 gene. Tumor cells thus modified present foreign peptide-derived epitopes in the form of processed peptides on their surface MHC molecules and are thus efficiently recognized by the immune system, leading to rejection also of unmodified, parental-type tumor cells.

We sought to utilize this mechanism for the generation of cancer vaccines by using synthetic, nonself, but MHC-matched peptides to bring about immunogenicity in whole tumor cells. Tumor cells displaying foreign peptides should then be efficiently recognized by the immune system and thus initiate an immune response against tumor-specific antigens. To test this concept, we chose the N-terminal influenza haemagglutinin peptide derivative LFEAIEGFI, a putative ligand for the MHC I haplotype H2-K^d. This allele is expressed on the murine melanoma M3 and colon carcinoma CT-26, two tumor cell lines used in the below animal models.

In order to elicit cellular immunity, the peptide is transloaded, i.e. it is applied to tumor cell in conjunction with polylysine- or transferrin/-polylysine/DNA complexes resulting in a foreign peptide transloaded vaccine (FPTV). To demonstrate that transloading is superior to simply pulsing cells with peptides, we performed FACS analyses of M3 cells which were transloaded, pulsed (long-term exposure to peptide in culture) or admixed (short-term exposure of peptide in cell suspension) with FITC-labeled peptide (**Fig. 5**).

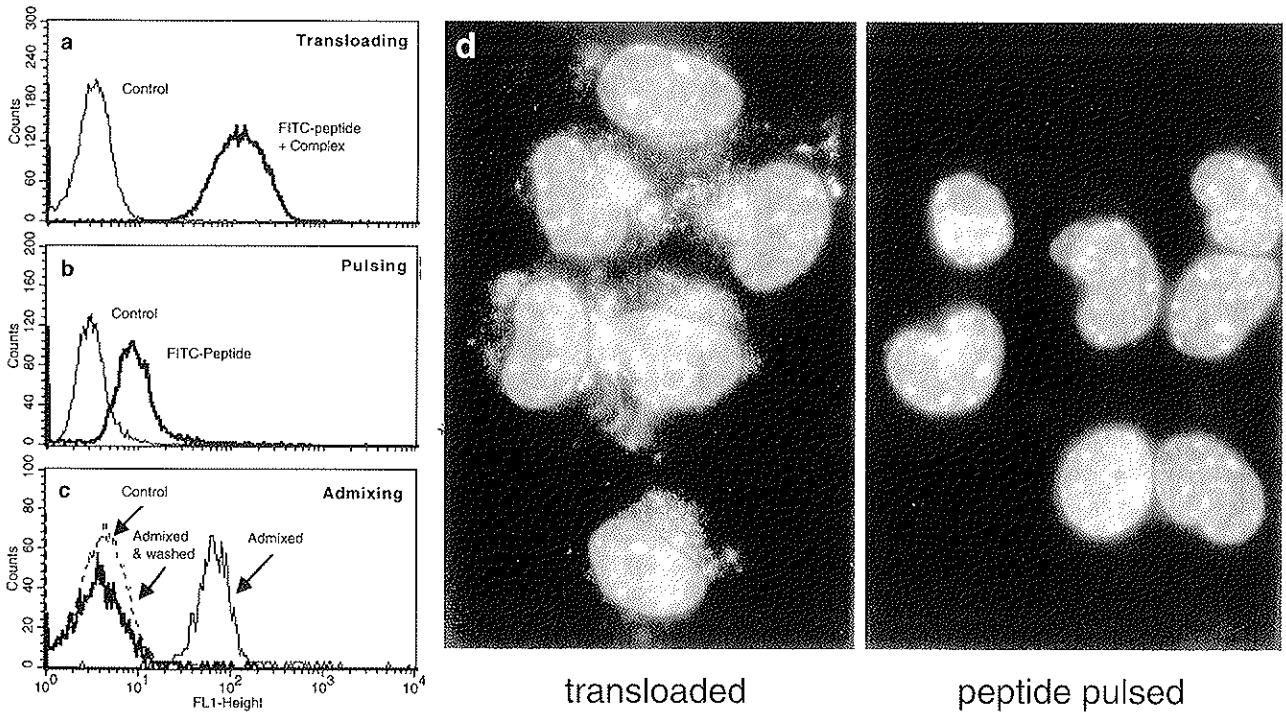

Fig. 5: FACS analysis of transloaded (a), pulsed (b) and admixed peptide (c). Immunofluorescence of transloaded and pulsed peptide (d).

After transloading, M3 cells showed an approx. two log shift in fluorescence as compared to control cells, a minor shift for pulsed cells and no such shift for admixed peptide, once the cells were washed. Specificity of peptide binding was verified by applying a peptide, whose anchor amino acids had been altered. This peptide was no MHC-I ligand and did not elicit anti-tumor immunity (see below) when transloaded on cancer vaccines.

Tumor cells transloaded with the MHC-I peptide of foreign origin were highly effective vaccines in two different murine tumor models, M3 and CT-26 colon carcinoma (see **Figs. 6a** and **6b**). In experiments where peptide transloaded vaccines were compared to IL-2 secreting vaccine, it became clear that peptide vaccines provided a better protection against tumor take. Pulsed or admixed peptides yielded poor vaccines, as most mice succumbed to tumors in these instances. No tumor protection could be obtained with the non MHC-I matched peptide.

Our study has identified a new general concept for the generation of highly efficient cancer vaccines consisting of whole tumor cells, transloaded with a synthetic, nonself peptide. It will be of interest to see whether this concept can be extended to include tumor antigen peptides and peptides derived from pathogens, which matched to the requirements of a desired MHC haplotype, might be applied for vaccination in other species inclusive of man.

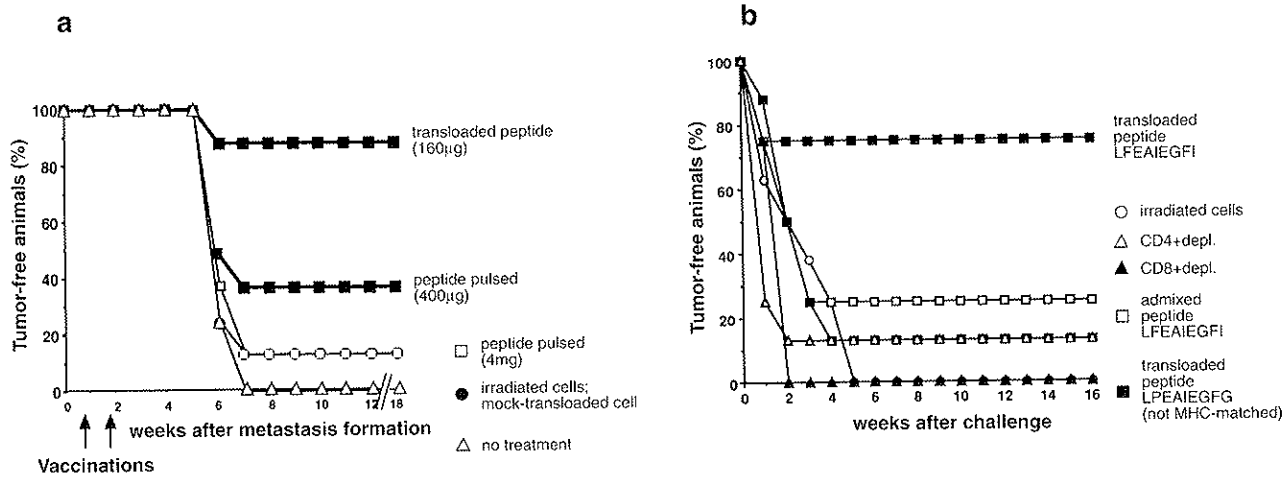

Fig. 6: Generation of anti-tumor immunity with antigenic peptide in DBA/2 mice bearing a subcutaneous M3 "micrometastasis" (a); in Balb/c mice challenged with CT-26 cells (b).

Publications during the year

Buschle, M., Cotten, M., Kirlappos, H., Mechtler, K., Schaffner, G., Zauner, W., Birnstiel, M.L. and Wagner, E. (1995). Receptor-mediated gene transfer into human T lymphocytes via binding of DNA/CD3 antibody particles to the CD3 T cell receptor complex. *Human Gene Therapy* **6**, 753-761.

Maass, G., Schmidt, W., Berger, M., Koszik, F., Schneeberger, A., Stingl, G., Birnstiel, M.L. and Schweighoffer, T. (1995). Priming of tumor-specific T cells in the draining lymph nodes after immunization with IL-2 secreting tumor cells: Three consecutive stages are required for successful tumor vaccination. *Proc. Natl. Acad. Sci. USA* **92**, 5540-5544.

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Leukemogenesis and carcinogenesis: Mechanisms in protooncogene- and oncogene function

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Staff scientist	Peter STEINLEIN
Postdoc	Marieke VON LINDERN (until Sept. 95)
Postdoc	Martin OFT
Postdoc	Kim C. LIM (until Oct. 95)
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PhD student	Oliver WESSELY
PhD student	Georg MELLITZER
PhD student	Anton BAUER
PhD student	Alexandra PACHER (since Oct. 95)
Technician	Evi DEINER
Technician	Lisbeth BOER (until Feb. 95)
Technician	Gabi STENGL (since Sept. 95)
Guest	Bianca HABERMANN (until Oct. 95)
Guest	Monika ANDERSSON (April -June 95)
Guest	Christine TRAN-QUANG (May-July and Sept.-Nov. 95)

Introduction and Overview

In 1995, our ongoing work on leukemogenesis in the chicken and mammary carcinogenesis in the mouse has increasingly focused on a number of new research areas that were initiated the year before (see Report 1994).

In our **first** system, leukemogenesis in chickens, we analyze how cooperation of receptor tyrosine kinases and nuclear steroid/thyroid hormone receptors regulates normal hematopoiesis. At the same time, we try to understand how the cooperation of two oncoproteins from the same gene families causes rapid, fatal erythroleukemia. These oncoproteins are v-ErbA and v-ErbB, expressed by the avian erythroblastosis virus (AEV), a retrovirus causing fatal erythroleukemia. v-ErbB is a truncated and mutated version of the avian epidermal growth factor receptor (TGFαR/c-*erb*B), that uses mammalian transforming growth factor (TGF) α as a ligand. V-*erb*A represents a mutated version of the thyroid hormone receptor α (TRα/c-*erb*A).

Previously, we reported that normal committed erythroid progenitors undergo prolonged self renewal (proliferation without apparent differentiation), if stimulated with combinations of receptor tyrosine kinase ligands (the c-Kit ligand stem cell factor, SCF and the c-ErbB ligand TGFα) and steroid hormones (estrogen; Hayman *et al.*, 1993; Schroeder *et al.*, 1993; Steinlein *et al.*, 1995). At least two progenitor types were identified. While SCF progenitors express the receptor tyrosine kinase c-Kit and, like the known erythroid progenitors BFU-E/CFU-E, are unable to undergo prolonged self renewal, SCF/TGFα progenitors express c-ErbB in addition to c-Kit and undergo sustained self renewal in response to the c-ErbB ligand TGFα and estradiol. SCF/TGFα progenitors develop from SCF progenitors, if the latter are exposed to a combination of SCF, TGFα, estradiol and an unknown factor from chicken serum. The identification of this additional factor was a major aim of last year's work (Oliver Wessely, Marieke von Lindern and Anton Bauer).

We also showed last year that the differential ability of c-Kit and c-ErbB to induce progenitor self renewal was both due to different developmental kinetics of receptor expression and to the activation of distinct, downstream signaling proteins by the two receptors (Wessely *et al.*, submitted). This year, our trials to identify distinct molecules differentially activated by the two receptors led to a detailed analysis of components of the Jak-Stat pathway (Georg Mellitzer and Oliver Wessely, together with M. Hayman, Stonybrook).

Furthermore, last year we established that self-renewing and differentiating erythroid progenitors differ in cell cycle- and cell size control. Self renewing cells (such as SCF/TGFα progenitors) "measure" their cell size at the so-called restriction point prior to entering S phase. The same cells fail to maintain a

constant size after differentiation induction, becoming smaller within each of five ensuing cell divisions. This seems to be due to a drastic shortening of the G1 phase of the "differentiation" cell cycle (Dolznig *et al.*, 1995). This year, we generated the prerequisites to start a molecular analysis of cell cycle control in self renewing and differentiating erythroblasts and report first results (H. Beug, together with H. Dolznig and E. Müllner, Vienna Biocenter).

Finally, we resumed our trials to determine how the v-ErbA oncoprotein may function in leukemogenesis. An important clue came from our observation in 1994, that the v-ErbA protein functionally replaced the endogenous estrogen receptor in self renewal induction together with endogenous receptor tyrosine kinases like c-ErbB and c-Kit. In 1995, we extended this analysis to other members of the steroid hormone receptor family, showing that v-ErbA affects the biological function of multiple members of the steroid/thyroid hormone receptor family in distinct ways (Anton Bauer and Marieke von Lindern, in cooperation with H. Stunnenberg, EMBL, Heidelberg, and B. Vennström, Stockholm).

In addition to work in these new areas, we continued to analyze oncogene function and cooperation in murine Friend leukemia using the chicken system. (Oliver Wessely and Hartmut Beug, in collaboration with Christine Tran-Quang and Jaques Ghysdael, Orsay, France). We also showed that the v-Ski oncoprotein, together with a ligand activated c-Kit receptor kinase, induced the self renewal of avian multipotent progenitors. Since these cells had an extended *in vitro* life span (100-110 generations) and differentiate into erythrocytes, mast cells, macrophages and probably neutrophils (Larsen *et al.*, 1993), we concentrated on this system, focusing on the cells´ requirements for self renewal on the one hand and on the factors determining their commitment and terminal differentiation on the other (H. Beug, P. Steinlein and O. Wessely, with Richard Dahl and M. Hayman, Stonybrook, New York).

The **second**, major interest of the laboratory is carcinogenesis, thought to be the result of multiple, cooperative genetic changes. We use mammary epithelial cells of the mouse that retain normal epithelial polarity, i.e. the ability to direct different proteins to the apical or the basolateral face of the epithelial cell in culture. In this system we analyze how single oncogenes involved in signal transduction (e.g. growth factor receptors, intracellular signaling proteins and transcription factors activated by intracellular signaling) affect *epithelial polarity* and *epithelial-fibroblastoid conversion*, i.e the regulated loss of epithelial polarity occurring during embryogenesis or tissue remodeling (Hay, 1990). Last year´s studies on the conditional AP-1 transcription factor c-JunER were concluded by their publication (Fialka *et al.*, 1995). In 1995, we concentrated on the question how the Ha-Ras oncoprotein (a constitutively active version of the c-Ras protein involved in receptor tyrosine kinase signaling) cooperates with endogenous transforming growth factor (TGF) β1 to induce epithelial-mesenchymal transition *in vitro* and *in vivo* (Martin Oft, in collaboration with Janos Peli and Ernst Reichmann, Lausanne).

A. Normal and leukemic erythropoiesis

1. The glucocorticoid receptor is a crucial player in induction and maintenance of committed erythroid progenitor self-renewal

Oliver Wessely, Marieke von Lindern, Evi Deiner and Hartmut Beug

Last year, we demonstrated that SCF progenitors (expressing c-Kit only and unable to self-renew) can develop into SCF/TGFα progenitors expressing c-ErbB and capable of continuous self renewal, if exposed to SCF, TGFα, estradiol and unknown factors from chicken serum. This was shown by limiting dilution cloning of SCF progenitors and following the fate of individual clones in the presence of various factor combinations, using chicken sera depleted for endogenous growth factors and hormones (Steinlein *et al.*, 1995).

First clues to the identity of this unknown factor(s) came from the use of media in which endogenous factors were depleted from the foetal calf serum as well as the chicken serum by charcoal treatment. In such media, SCF/TGFα progenitors neither developed nor proliferated when exposed to SCF, TGFα and estradiol. Addition of hydrocortisone or the synthetic glucocorticoid receptor (GR) ligand dexamethasone (Dex) fully restored the ability of the cells to grow. Also, the development of SCF/TGFα progenitors from SCF progenitors proceeded with maximum speed when SCF, TGFα, estradiol and Dex were provided, but no such development occurred in the absence of Dex. Most likely, Dex was the major activity required, since addition of a specific GR antagonist (ZK 112993) completely inhibited both development and proliferation of SCF/TGFα progenitors in media containing untreated sera.

To analyze whether the activated GR would also arrest erythroid differentiation, the effect of Dex was analyzed in erythroblasts under differentiation

conditions (Epo/Ins) or upon delayed differentiation induced by SCF plus Epo/Ins. Interestingly, addition of Dex alone had essentially no effect. However, Dex in combination with SCF induced a strong arrest of differentiation and resulted in continuous self renewal of the progenitors. The latter process did not require the presence of c-ErbB, since it occurred in the presence of a specific inhibitor of the c-ErbB receptor kinase (PD 153035, Fry *et al.*, 1994). Also, complete inhibition of differentiation by Dex plus SCF required the presence of estradiol, which suggests that the two steroid hormones do not have identical, but rather complementary functions. Currently, we investigate why these progenitors are apparently able to self renew in the absence of an active c-ErbB. A likely possibility is that the activated Epo-receptor may replace c-ErbB function (see below).

Since the GR is known to mediate transcriptional activation as well as repression, the question arose which of these putative mechanisms may be more important for GR function in self renewal induction and differentiation arrest. The partial GR-agonist RU486 mediates nuclear transport and subsequent transrepression by GR, but prevents transcriptional activation. In erythroid progenitors, RU486 acted as a complete Dex antagonist, which indicates that transcriptional repression by the GR is not sufficient to sustain self renewal. To test more directly whether gene regulation involving specific binding of the GR to cognate glucocorticoid responsive elements (GRE´s) does play a major role in self renewal, a mutant human estrogen receptor (HE82), carrying three point mutations in the P box of the DNA binding domain (E 57-G; G58-S; A 61-V, numbering of amino acids as in v-ErbA) was expressed in primary progenitors. Because of its P box mutations, the DNA binding specificity of HE82 is altered, specifically binding to GRE´s rather than to estrogen responsive elements (ERE´s).

To our surprise, erythroid progenitors expressing this HE82 construct underwent continuous self renewal in the complete absence of Dex (i.e. in SCF, TGFα, estradiol and the Dex antagonist ZK112993), while control cells or cells overexpressing a murine ER were totally dependent on Dex for self renewal under the same conditions. Additionally, the block of differentiation induced by SCF, dexamethasone, estradiol and Epo/Ins in control cells could be maintained in the HE82 cells by SCF, estradiol, Epo/Ins and ZK112993 (**Fig. 1**). This clearly demonstrates that transcriptional regulation via specific DNA binding by the GR is essential for the observed effects of dexamethasone on erythroid self renewal and differentiation.

To assay for genes whose expression could be regulated by an activated GR, Northern blot and run-on analyses are currently being performed. A first result from these ongoing studies is that GR activation seems to activate c-*myb* expression independent of the presence of SCF, whereas repression of α-globin transcription requires the presence of both dexamethasone and SCF.

The observed cooperation between SCF and dexamethasone could mean that phosphorylation of the GR via SCF-activated signal transduction is required for efficient transcriptional activation. However, transactivation assays using reporter constructs harbouring a GRE showed no effect of SCF on the transactivation capacity of the GR in primary erythroid cells. Therefore, at present it seems more likely that SCF and dexamethasone activate parallel pathways in the cell. In cooperation, these pathways may cause self renewal rather than differentiation in erythroid progenitors.

In summary, our results show that regulation arrest of self renewal and differentiation in normal erythroid progenitors is even more complex than previously anticipated, now involving two steroid hormone receptors (ER/GR) as well as at least two (receptor) tyrosine kinases (c-Kit plus c-ErbB or EpoR/Jak complexes).

2. Different types of normal erythroid progenitors can be distinguished by their cell surface antigens

Peter Steinlein and Evi Deiner

A major open question with respect to normal, avian erythroid progenitors is whether the different types described (SCF progenitors, SCF/TGFα progenitors, progenitors growing in the above factors plus Dex) exhibit other differences than varying levels of c-ErbB expression (Steinlein *et al.*, 1995) and whether the different progenitor types (particularly the c-ErbB expressing ones) are present under physiological conditions in bone marrow. To answer this question, molecular differences between these types of erythroid cells need to be identified that would allow identification and subsequent isolation of the cells from untreated bone marrow.

The approach chosen was to characterize the various progenitor types for cell surface antigens, using fluorescent monoclonal antibodies in combination with FACS analysis and subsequent cell sorting. In contrast to the mammalian system, only few antibodies recognizing avian hematopoietic cells have been described. However, about 25 monoclonal antibodies raised to chicken hematopoietic cells are available in our laboratory, which so far have only

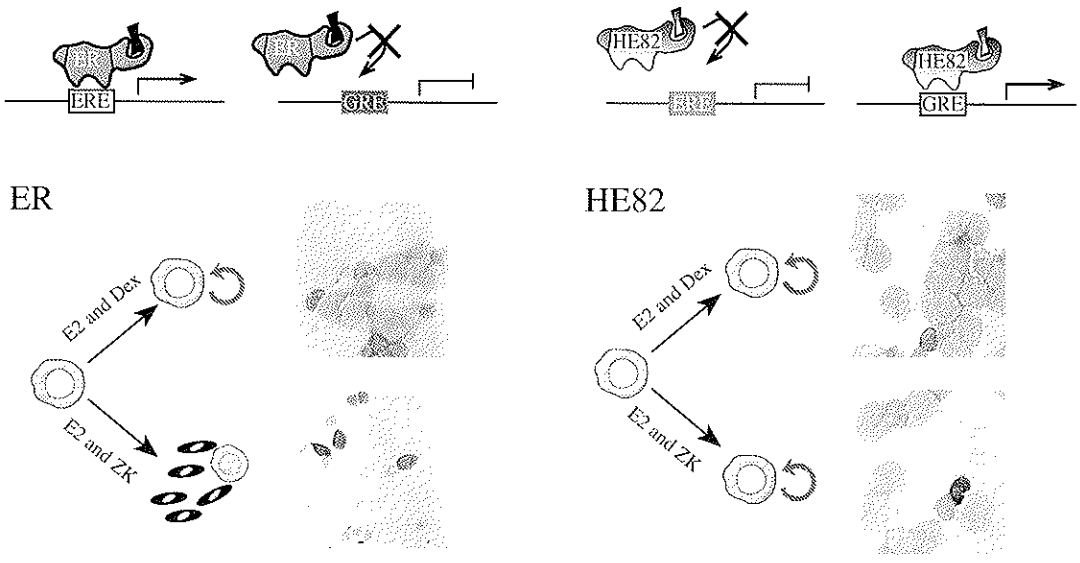

Fig. 1: A mutant ER with GR-binding specificity induces erythroblast self renewal. To evaluate the contribution of transcriptional regulation via specific DNA elements to the phenotype induced by an activated GR, a mutant human estrogen receptor (HE82) which specifically recognizes a GRE instead of an ERE (top) was expressed in primary avian erythroblasts. Control erythroblasts expressed empty vector or wild-type (wt) ER. The cells were then induced to differentiate in the presence of Epo/Ins, SCF and either estradiol and dexamethasone (E2 and Dex) or E2 plus Dex antagonist (E2 and ZK). After 5 days, cells were cytocentrifuged onto slides, stained with neutral benzidine plus histological dyes and photographed under blue light to reveal histochemical staining for hemoglobin (dark appearance of cells). While the cells containing the empty vector control as well as wtER require Dex (i.e. an active GR) for proliferation and differentiation arrest, HE82 effectively stimulated proliferation and arrested differentiation in the absence of an active GR.

been characterized by conventional immuno-fluorescence or not at all. In a first set of experiments, seven of these antibodies have been characterized in detail with respect to their cell specificity. To analyze the different erythroid progenitor types, bone marrow cells were cultivated in various mixtures of hormones and growth factors and the outgrowing cells repeatedly analyzed with the above panel of antibodies.

Several antibodies were found which yielded useful differences in surface staining between SCF- and SCF/TGFα progenitors on the one hand, and between erythroid progenitors grown in the presence or absence of glucocorticoids on the other. Interestingly, all these antibodies were raised against very immature cell types (primary or established, multipotential cells transformed by the E26 avian myeloblastosis virus). The most significant observation was that Dex treatment induced a high proportion of the cells to express antigens detected by the above antibodies, while SCF progenitors (and erythroblasts transformed by leukemogenic avian retroviral oncogenes like v-*erb*B) hardly expressed these antigens.

We then used these antibodies, together with fluorescently labeled transferrin to detect high transferrin receptor levels as a general erythroid marker, to sort erythroid cells from non purified bone marrow cultures after 1-3 days (containing 5-30% erythroid cells at all stages of maturation). If seeded under optimal conditions for self renewal induction (SCF, TGFα, Estrogen, Dex, see above) a very high proportion of the sorted cells proliferated. We are currently trying to use this method to determine the presence and abundance of cells able to self renew in fresh, untreated bone marrow and to bone marrow treated with different factor combinations for 1-2 days.

3. Factor/hormone combinations inducing prolonged self renewal in avian erythroblasts can do so also in primary human erythroid progenitors

Marieke von Lindern, Peter Steinlein and Hartmut Beug, with W. Zauner (IMP), W. Fritsch (St. Anna Children's Hospital, Vienna) and K. Huber (AKH, Vienna).

Our results in chicken erythroblasts showed that committed erythroid progenitors can be induced to sustained self renewal by certain combinations of growth factors and steroids. This clearly violated a dogma in hematopoiesis, that only the pluripotent stem cell can self renew (Keller, 1992). The same result, however, made it easier to explain aberrant self renewal of committed progenitors occurring in leukemia (for review see Sawyers *et al.*, 1991). A more comprehensive analysis of erythroid commit-

ment and differentiation would require the combination of our avian *in vitro* culture systems with the analysis of mice in which genes involved in erythropoiesis have been disrupted, and with the analysis of normal and aberrant erythropoiesis in humans. It was therefore highly desirable to determine whether self renewal of committed progenitors would also occur in mammalian hematopoiesis. The long *in vitro* life span of human cells (50 -70 doublings, in contrast to 7-15 in murine cells) was our major reason to first try these experiments using human erythroid progenitors.

As a source for such progenitors, CD34 positive cells isolated from neonatal cord blood were used. Pilot experiments had shown that human erythroid progenitors could be grown for >10 divisions in the presence of recombinant human erythropoietin (rhEpo), mammalian SCF and estradiol. These human erythroblasts were absolutely dependent on both rhEpo and SCF. The rhEpo appears to be the main proliferation stimulus, whereas the presence or absence of SCF determines whether the cells self-renew or differentiate. SCF alone did not or only weakly induce cell proliferation, but synergized strongly with the mitogenic effects of rhEpo. IGF1 appeared to function very similarly to SCF.

Our above results on the action of the GR in avian erythroid progenitors prompted us to try similar factor combinations in human cells. As in the chicken system, activation of the GR resulted in a drastically enhanced self renewal capacity of human erythroid progenitors. In the presence of rhEpo, SCF, estradiol and dexamethasone (Dex), erythroid progenitors underwent self renewal for 20-25 generations, resulting in a 10^5-10^6 fold net increase in the number of erythroid cells. Cells treated with Dex maintained high levels of c-Kit throughout their life span and were much more effectively arrested in differentiation. Colony assays in semisolid medium and FACS analysis for a large number of lineage markers showed that the mass cultures obtained under these conditions contained 80-95% of immature progenitors committed to the erythroid lineage. The fact that a majority of the cells (60-80%) expressed high levels of c-Kit (CD117), together with the results of the colony assays suggests that many of these progenitors correspond to the BFU-E type of erythroid progenitors.

The second erythroid progenitor found in the chicken, i.e. SCF/TGFα progenitors, could not yet be demonstrated in the human system. Despite testing a large variety of receptor tyrosine kinase ligands, human progenitors with corresponding properties (i.e. surviving for 50-70 generations and expressing c-ErbB or related receptor tyrosine kinases) have not yet been observed. It is still unclear whether this is due to a genuine species difference, to a shorter in vitro life span of human erythroblasts as compared to fibroblasts, or to the fact that suitable erythroid progenitors able to undergo this development (bone marrow cells from very young individuals) are just not available from human sources.

Having established that self-renewing, human erythroid progenitors can indeed be obtained, we analyzed the role of various factors during terminal differentiation of these human erythroid progenitors (see **Fig. 2**). In the presence of recombinant human erythropoietin and insulin, differentiation into enucleated erythrocytes was reproducibly obtained. At the onset of terminal differentiation, c-Kit and the IGF1 receptor are downregulated while expression of the insulin receptor increases. Insulin and rhEpo cooperate during terminal differentiation. Differentiation is retarded by SCF or IGF1 and accelerated by activation of the thyroid hormone receptor or the retinoic acid receptor. Interestingly, TGFβ did not inhibit cell proliferation, but induced differentiation under self renewal conditions (**Fig. 2**).

In conclusion, it is possible to induce prolonged self renewal in normal human erythroid progenitors, similar to self renewal in avian progenitors. Initial experiments suggest that these human erythroid progenitors are suitable for gene transfer studies. Thus, *in vitro* studies of aberrant erythropoiesis in man are feasible and may allow approaches to correct defects by gene transfer. In addition, pilot experiments suggest that we will be able to grow and analyze fetal liver-derived murine erythroid cells *in vitro* using similar hormone mixtures, thus allowing to determine the effects of targeted gene disruptions on murine erythroid self renewal and differentiation.

4. Cell cycle- and cell size control in self renewing and differentiating erythroblasts

Hartmut Beug and Evi Deiner, with H. Dolznig and E. Müllner (Biocenter Vienna), K. Nasmyth (IMP) and H. Kiyokawa (Sloan Kettering Res. Inst., USA)

Last year, we demonstrated that normal chicken erythroid progenitors drastically switch their gene expression program between 16 and 24 hours after differentiation induction. At the same time, cell cycle parameters are reprogrammed in a developmentally controlled process, involving shortening of G1 from 12 to 5 hours and progressive reduction of cell size from 350 to 80 femtoliters. These results suggested that differentiating SCF progenitors may indeed exhibit fundamentally altered cell cycle control, in which cell size was either not controlled at all, or according to a different program.

To analyze potential differences between "self renewal divisions" and "differentiation divisions" (see

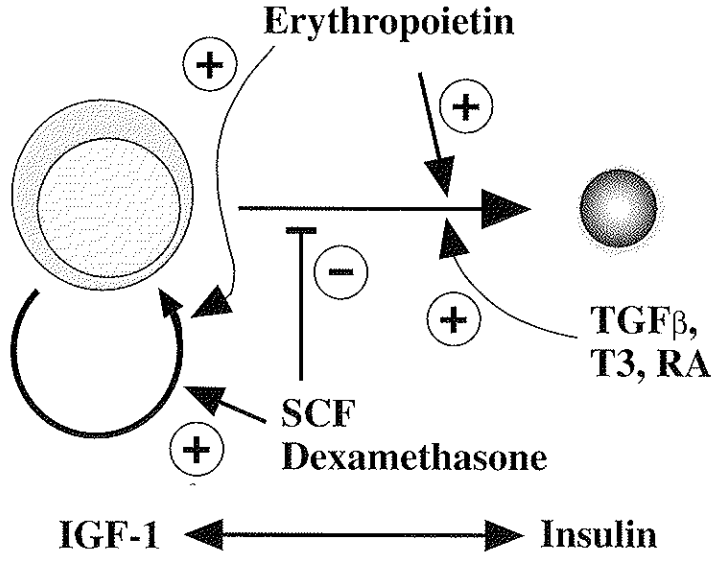

Fig. 2: Regulation of self renewal and differentiation in human erythroid progenitors by growth factors and hormones. This simple scheme depicts how various growth/differentiation factors and steroid hormone receptors act and cooperate in regulating self renewal and differentiation of normal human erythroid progenitors. While SCF, Epo and Dex cooperate in stimulating (+) proliferation of immature erythroid cells (erythroblast symbol with circular arrow, left), SCF and Dex also inhibit (-) differentiation of these progenitors (erythrocyte symbol, right). Erythropoietin alone also stimulates (+) differentiation, as do transforming growth factor (TGF) β, thyroid hormone (T3) and retinoic acid (RA). While IGF-1 (and its receptor) functions mainly in immature cells, the main role of insulin is during terminal differentiation (horizontal double arrow).

Fig. 3) at the molecular level, several prerequisites had to be fulfilled. We already described in 1994 that pure fractions of cells at given phases of the cell cycle could be produced by centrifugal elutriation. Starting from 500-800x10^6 cells, 75-95% pure fractions of viable cells in G1, late G1, S, early and late G2 and in mitosis could be obtained. These fractions contained enough cells (20-50x10^6) to allow biochemical and molecular analyses. However, pilot experiments showed that mRNA expression of certain cell cycle components (e.g. A and B cyclins) was affected by the elutriation procedure and that a variable fraction of the cells tended to enter apoptosis due to the lack of growth factors during elutriation.

These problems have now been overcome by altering the media used for elutriation and by recultivating the elutriated cell fractions in their original growth or differentiation media for 6-8 hours. In such cells, the synchrony of cell cycle phases in the different fractions is fully maintained and the fractions now show the expected patterns of cyclin mRNA expression. In addition, these elutriations allowed us to clearly demonstrate that the rate of size increase was constant in self-renewing and differentiating erythroid progenitors and that the decrease in size observed in the differentiating cells was fully accounted for by the shortened G1 phase (**Fig. 3**).

As a second prerequisite, molecular probes for important components of the cell cycle machinery [cyclin-dependent kinases (cdk´s), cyclins, regulatory components such as cdk-inhibitors etc.] had to be obtained. Unfortunately, only cdk-1 and the G2/M cyclins A and B1, B2, B3 were available as chicken c-DNA´s. To clone the more important G1 and S cyclins (cyclins D1, D2, D3 and E) as well as other cdk´s (cdk-2, cdk-4 etc.) a strategy for cloning the respective chicken genes was developed, involving a comparison between the mammalian and *Xenopus* genes and designing appropriate conserved PCR primers. By this method, partial clones of chicken cyclins D1 and D2, as well as cdk-4 could be isolated and verified by sequencing. In the meantime, chicken cyclin E and cdk-2 were cloned by another group and could be obtained from this source.

Next, these probes were used to determine the expression kinetics of cyclins and cdk´s typical for the G1, S and G2M phases during differentiation. Self-renewing erythroid progenitors or the same induced to differentiate for 16-120 hours were processed for RNA isolation and Northern blot analysis . The blots were then analyzed with the various c-DNA probes. Interestingly, cyclins D1 and D2 mRNA´s as well as cdk-4 mRNA were almost completely downregulated between 24 and 48 hours after differentiation. In case of cyclin D1 and cdk-4, this downregulation could be confirmed at the protein level, using Western blot analysis with suitable antibodies. In contrast, cyclins A, B1, and B2 mRNA´s as well as cdk1 and cdk2

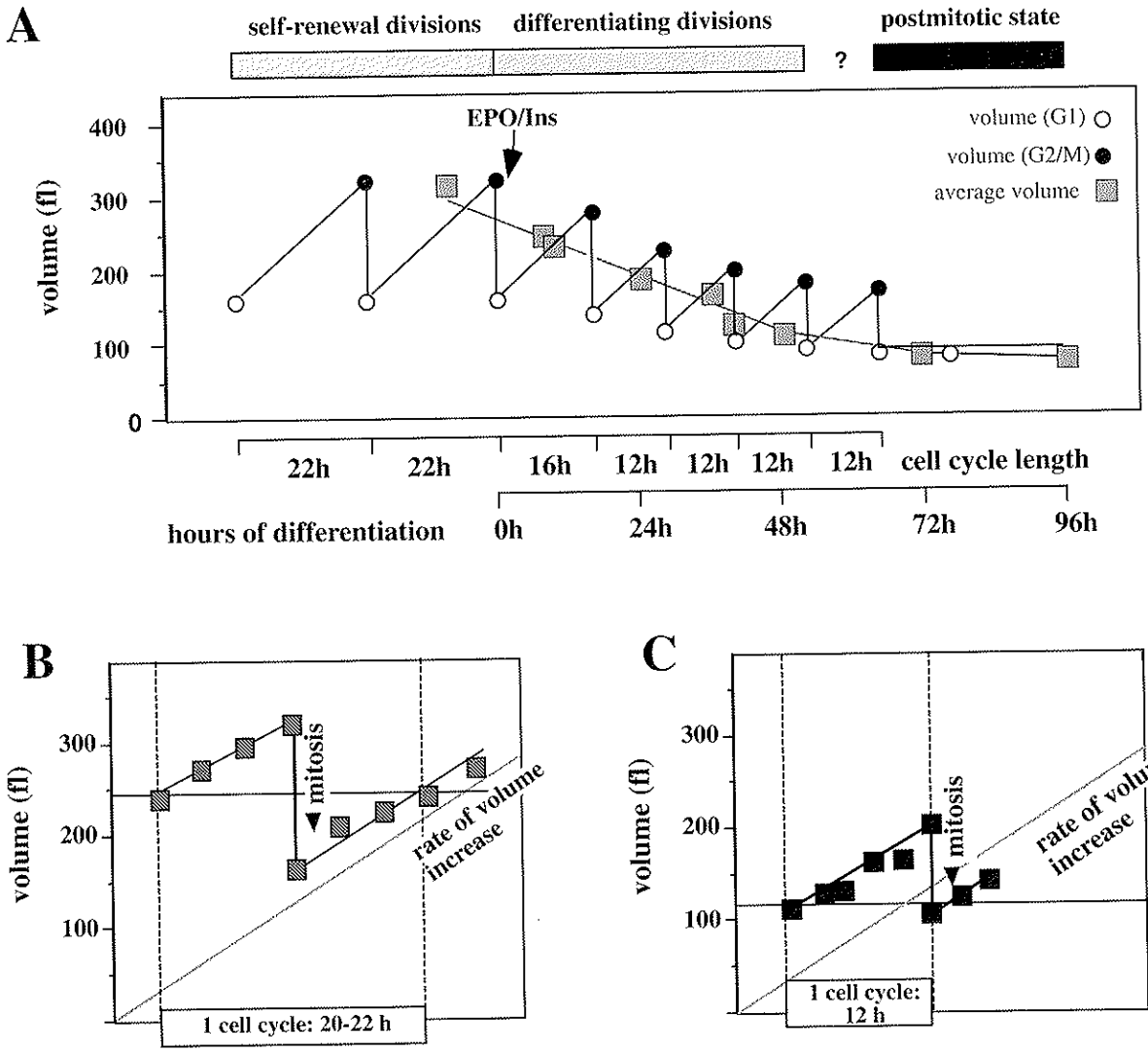

Fig. 3: Shortening of G1 fully accounts for cell size reduction in differentiating erythroid progenitors: Uncoupling of cell size control from cell cycle progression.
(A). *Size reduction model for differentiating avian erythroblasts.* Assuming that the rate of cellular mass increase between early G1 (open circles) and late G2 (closed circles) is constant in self renewing and differentiating cells, the kinetics of cell volume changes during self renewal divisions (lightly shaded bar) and differentiation divisions (darkly shaded bar) can be calculated. The mean cell sizes calculated (hatched line) correspond very well to cell sizes actually determined (cross-hatched squares).
(B, C). *Verification of the model shown in (A.)* Self renewing erythroblasts (B; hatched squares) and cells induced to differentiate for 48 hours (C; dark squares) were subjected to centrifugal elutriation and the fractions obtained recultivated for another 8 hours to allow late S and G2 fractions to go through mitosis. Thereafter, the fractions were subjected to cytofluorometric determination of DNA content to determine cell cycle phase (see legend to Figure 4) and their size was measured in an electronic cell counter (CASY, Schärfe Systems). As postulated in (A), the rate of volume increase during cell cycle progression (hatched lines)was the same in self-renewing and differentiating cells.

mRNA´s were first upregulated during the first 24-48 hours of differentiation induction and only lost after 72-90 hours, when the cells became postmitotic (Dolznig *et al.*, 1995).

The behavior of cyclin E was also interesting. Although its messenger RNA was downregulated relatively early (36-50 hours) after differentiation induction, cyclin E protein levels behaved like those of the G2/M cyclins, i.e. they remained constant until 72-90 hours after differentiation. In conclusion, shortening of G1 occurring 16-24 hours after differentiation induction is correlated with expression-reduction of G1-phase-specific regulators (cyclins D1,D2, cdk-4), while expression of S/G2-specific regulators is maintained. To a certain extent, this is reminiscent of the cell cycle regulation in early embryos, where no G1 phase exists (and thus no G1 cell cycle regulators are expressed) and where cyclin E expression is maintained at constant, high levels. Interestingly, these early, embryonic cleavage divisions involve no measurement or control of cell size.

5. Self renewal induction: c-ErbB but not c-Kit activates a STAT 5b-like factor in avian erythroid progenitors.

Georg Mellitzer, Oliver Wessely and Hartmut Beug, with T. Decker (Vienna Biocenter) and M.J. Hayman (Stonybrook, USA)

In pursuit of the question, which molecular mechanisms are responsible for the fact, that c-ErbB can induce sustained self-renewal in erythroid progenitors, while c-Kit is unable to do so, first evidence obtained in 1994 suggested that receptor-specific signal transduction was involved. When avian c-ErbB and human c-Kit were overexpressed by retroviral vectors in cells lacking endogenous c-ErbB, the exogenous c-Kit failed to cause self renewal while c-ErbB did. Indirect evidence for the notion that c-Kit and c-ErbB indeed activated different downstream signal transduction proteins was obtained by use of specific tyrosine kinase inhibitors which did not affect ligand-induced receptor autophosphorylation (tyrphostins). One of these inhibitors (AG30) specifically blocked self renewal induced by c-ErbB, while a second one (AG 776) preferentially inhibited c-Kit function (O. Wessely *et al.*, manuscript submitted).

In 1994, we also obtained first evidence that members of the STAT (**S**ignal **T**ransduction **A**ctivated **T**ranscription factor) family of transcription factors were preferentially activated by c-ErbB. STAT´s become phosphorylated on tyrosine by ligand activation of receptor tyrosine kinases or cytokine receptor-Jak complexes. This then causes dimerization of STAT´s, translocation to the nucleus, binding to specific DNA-elements and regulation of transcription of respective target genes. In 1995, STAT activation by c-ErbB, c-Kit and other receptors expressed in normal erythroid progenitors was analyzed in more detail.

An important prerequisite for these studies were cells and/or conditions which allowed to completely turn off signal transduction by c-Kit or c-ErbB and then activate the receptors for short time periods by ligand addition. Since this turned out to be difficult in primary erythroblasts, we made use of a derivative (HD3EpoR-E22) of the avian erythroblast cell line HD3. This recently developed cell line stably expressed the murine Epo receptor and could be induced to synchronously differentiate in the presence of human recombinant Epo and Insulin, when the oncogenes v-ErbB (and v-ErbA) were completely turned off by shift to 42°C plus inhibition of residual v-ErbB kinase activity by the specific inhibitor [PD 153035, (Fry *et al.*, 1994)]. These cells also expressed endogenous, bioactive c-Kit and c-ErbB and allowed complete silencing of receptor activity by prolonged incubation in serum-free media.

We first assayed if tyrosine phosphorylation of STAT´s by c-ErbB would activate them to bind to specific STAT-DNA recognition sequences. In gel-retardation assays, we tested different GAS-elements for their ability to bind activated STAT´s. These analyses indicated that the c-ErbB activated STAT-proteins bound with high affinity to an IFP-53-GAS element, while three other elements were only bound with low affinity or not at all. No binding of STAT proteins to DNA was induced after ligand activation of c-Kit.

To identify the STAT transcription factor(s) present in these STAT/DNA complexes, several antibodies specific for different STAT proteins (STAT1, STAT3, STAT5a and STAT5b) were analyzed for their ability to react with these complexes. Of the tested antibodies, only the STAT5b specific antibody induced a super-shift of the c-ErbB activated STAT-complex. Using reporter plasmids containing 2, 3 or 6 IFP53-GAS elements together with a minimal promoter, we also could show that STAT5 was able to transactivate such reporter genes after being activated by c-ErbB. Out of several other growth factors tested for activation of STAT5 in avian and human erythroblasts, only Epo was able to induce STAT5 binding to DNA, but unlike TGFα failed to cause transactivation of respective reporter genes (see **Fig. 4**).

To analyze, if STAT5b (and/or other STAT proteins) were indeed phosphorylated on tyrosine, lysates of SCF/TGFα progenitors were immunoprecipitated by various antibodies to STAT proteins and the immunoprecipitates analyzed by Western Blot using phosphotyrosine-specific antibodies. Firstly, a p91/84 STAT protein doublet recognized by a polyvalent STAT antibody was differentially phosphorylated by c-ErbB and c-Kit. Both the 91kd and 84kd proteins were tyrosine-phosphorylated after activation of the c-ErbB, while c-Kit seemed only to phosphorylate the 84 kd protein. Secondly, selective phosphorylation by c-ErbB was also found for a 90-95 kd protein doublet recognized by a STAT5b antibody.

Taken together, one signaling pathway specifically activated by c-ErbB could be identified, which involved STAT5b homodimers. An investigation of Ras-pathway components (Shc, c-Ras) as well as of the known c-Kit signaling intermediate p85 PI-3 Kinase for activation by c-ErbB and c-Kit is in progress.

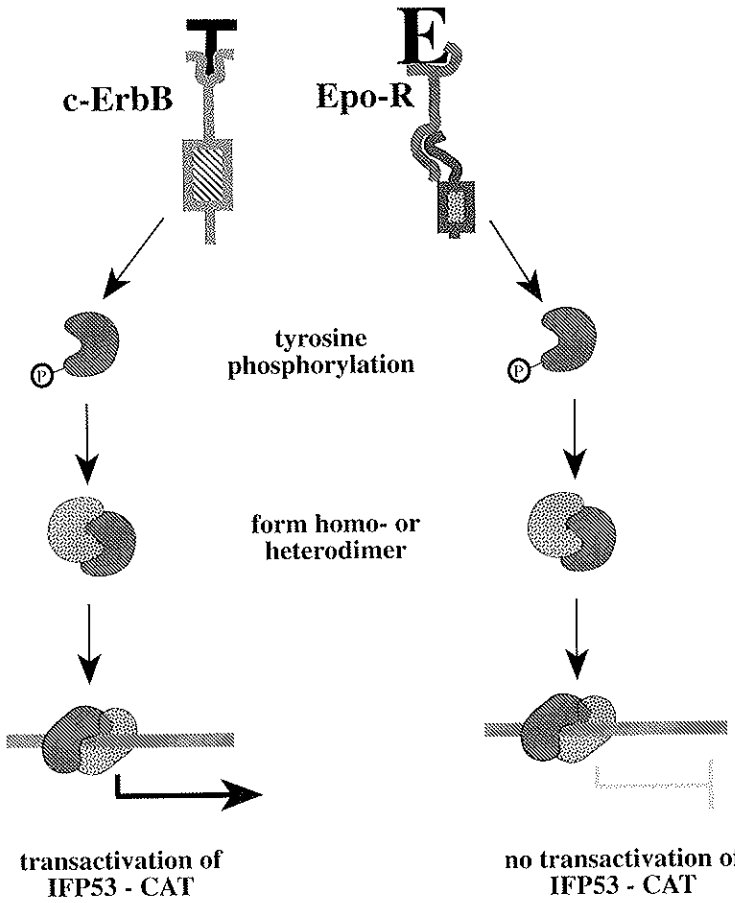

Fig. 4: Activation of STAT 5b by c-ErbB and EpoR in erythroblasts: Differential effects on transactivation
This simple scheme depicts our current view how endogenous receptors expressed in self renewing erythroid progenitors activate components of the Jak-STAT pathway. While c-ligand (T) activated ErbB (left pathway) specifically causes tyrosine phosphorylation of STAT5b, as well as its heterodimerization, binding to specific DNA elements and transactivation of reporter genes (IFP53-CAT), the ligand (E) activated Epo-receptor (EpoR, right pathway, bound to a Jak tyrosine kinase) activates the STAT5b pathway to the stage of specific DNA binding, but fails to induce transactivation.

6. V-ErbA oncoprotein function: Replacement of steroid hormone receptor function and cooperation with ligand-activated receptor tyrosine kinases

Anton Bauer, Marieke von Lindern and Hartmut Beug, with H. Stunnenberg (Heidelberg) and B. Vennström (Stockholm)

Last year, we demonstrated that v-ErbA, a mutated version of the avian thyroid hormone receptor α (c-ErbA/TR-α), required the cooperation with endogenous, activated receptor tyrosine kinases such as c-Kit or c-ErbB to cause erythroblast self renewal and arrest erythroid differentiation. Similar to the steroid hormone receptors ER and GR, v-ErbA was totally inactive in the same cells, if no activated receptor tyrosine kinase was present. This raised the possibility that v-ErbA would act by replacing distinct, biological functions of one or both of these steroid hormone receptors. First evidence for this notion was obtained by showing that v-ErbA-induced self renewal of normal erythroid progenitors in the presence of an estradiol antagonist, ICI164384. Furthermore, evidence was obtained that the estrogen receptor indeed retarded erythroid differentiation and caused repression of certain erythrocyte genes via a DNA-binding independent mechanism (see Report 1994).

This year, we concentrated on the questions (i) if v-ErbA could also replace biological functions of the glucocorticoid receptor, (ii) if specific DNA binding and/or heterodimerization to the co-receptor RXR were important for v-ErbA function and (iii) if phosphorylation of v-ErbA (shown to be required for ErbA function in cooperation with oncogenic receptor tyrosine kinases like v-ErbB or v-Sea; Glineur *et al.*, 1990) was important for v-ErbA cooperation with c-Kit in normal erythroid progenitors. The latter two questions were addressed by means of respective v-ErbA mutant proteins.

V-erbA replaces GR function in normal progenitors

To investigate whether v-ErbA can replace not only estradiol, but also dexamethasone (Dex) in erythroid progenitor self renewal, v-ErbA-expressing chicken bone marrow cells were cultivated in the presence of either SCF or TGFα, together with an estrogen antagonist (ICI 164384), a glucocorticoid antagonist (ZK 112993) or both. Surprisingly, the v-ErbA expressing cells underwent sustained self renewal even in the presence of both antagonists. Indeed, the growth rate of the v-ErbA expressing cells was comparable under all conditions or even enhanced by the Dex antagonist. This strongly suggests that v-ErbA can indeed functionally replace both the ligand activated ER and GR in erythroid self renewal. As expected, control cells infected with empty vector only failed to proliferate in the presence of either antagonist. Interestingly, the v-ErbA expressing cells were even able to grow in the presence of the above steroid antagonists plus the specific c-ErbB inhibitor PD 153035. Thus, cooperation of c-Kit with v-ErbA is sufficient for prolonged self renewal and an active c-ErbB is not required. Likewise, v-ErbA was able to completely arrest differentiation in the presence of both antagonists, when cooperating with a ligand-activated c-Kit. Our results clearly demonstrate that v-ErbA replaces certain biological functions of the ER and the GR, required for either self renewal induction and/or for arrest of terminal differentiation.

Dimerization domain mutants

V-ErbA is currently thought to act as a dominant negative c-ErbA/TR-α (Sap *et al.*, 1989) but is unable to heterodimerize with the co-receptor RXR. Since dominant negative retinoid receptors were recently shown to require an intact RXR heterodimerization domain (Durand *et al.*, 1994), we compared v-ErbA to a v-ErbA mutant (v-ErbA-r12) that has regained the ability to heterodimerize with RXR. When v-ErbA-r12 was expressed in SCF progenitors and compared to wild-type v-ErbA, it supported the sustained self renewal of erythroid progenitors in the presence of SCF plus estradiol/Dex antagonists like v-ErbA-wt, even causing a much faster onset of sustained proliferation than v-ErbA. To analyze if the gain of RXR heterodimerization ability in v-ErbA-r12 would enhance its ability to repress the growth-inhibiting, differentiation-inducing effects of ligand activated c-ErbA/TR-α or RARα (Schroeder *et al.*, 1992a; Schroeder *et al.*, 1992b), cells expressing wt-v-ErbA or v-ErbA-r12 were induced to differentiate in the presence of SCF, receiving or lacking thyroid hormone (T3) plus 9-cis retinoic acid (9cRA). This treatment activates both c-ErbA- and RARα heterodimers with RXR.

In the absence of RA/T3, both v-ErbA-wt and v-ErbA-r12 showed the expected, complete arrest of differentiation. Surprisingly, however, T3 plus 9cRA were clearly able to partially overcome the ErbA/c-Kit induced differentiation arrest in the v-ErbA-wt expressing cells. In contrast, T3 plus 9cRA had no detectable effects on v-ErbA r12 expressing cells under the same conditions. These data indicate that an intact RXR heterodimerization interface is indeed required to convert v-ErbA into a truly dominant-negative receptor, now being able to completely suppress the activity of both ligand-activated, endogenous TR and RAR. Obviously, v-ErbA-wt can only be envisaged to function as a dominant-negative receptor by either acting as a homodimer or by forming low-affinity v-ErbA/RXR complexes due to extreme overexpression of the v-ErbA protein.

A region in the v-ErbA P-box is crucial for v-ErbA function

If specific DNA binding would be required for v-ErbA to replace functions of the ER or GR, the mutations in the v-ErbA DNA binding domain should alter or relax the DNA binding specificity relative to c-ErbA. Two mutations of v-ErbA in the P box (determining DNA binding specificity) have been described (Bonde *et al.*, 1991). While an S61-G mutation (representing the c-ErbA configuration) failed to bind an artificial TRE (TRE-pal) and reduced transforming activity, a second mutation at the same position (S61-A, corresponding to the sequence of the ER at this position) neither affected TRE-pal binding nor transforming activity.

To analyze the effect of these P-box mutations on v-ErbA activity in normal erythroid progenitors, the S61-G- or S61-A mutant v-ErbA proteins were tested for their ability to induce self renewal in SCF plus ICI 164384 and/or ZK 112993. Surprisingly, in the absence of steroids, the Ser to Gly backmutation (S61-G) completely abolished the ability of v-ErbA to cooperate with c-Kit in supporting erythroid self renewal. In contrast, the S61-A v-ErbA protein was superactive, strongly increasing the ability of v-ErbA to cause long-term progenitor proliferation.

Similar results were obtained by analyzing erythroblasts expressing the two mutant v-ErbA proteins

for their differentiation phenotypes in presence of Epo/Ins plus SCF. V-ErbA S61-G was essentially unable to arrest differentiation in cooperation with c-Kit (except for reduced hemogobin content). In contrast, the S61-A mutation was superactive also in causing a differentiation arrest, since it did partially inhibit maturation even in the absence of a ligand-activated c-Kit (particularly evident again for hemoglobin accumulation). Taken together, our data show that the domain of v-ErbA surrounding serine 61 must be of crucial importance for v-ErbA function.

When analyzed for the ability to repress erythroid genes, the S61-G mutant behaved like a defective v-ErbA with respect to the three known v-ErbA target genes (CAII, Band 3 and Ala-S). Interestingly, however, the S61-G mutant was able to maintain high c-Myb mRNA levels for more than 6 days, at which time all cells were either completely or partially mature. This maintenance of c-Myb may be relevant for the relatively low hemoglobin levels present in the differentiated cells as well as for their somewhat aberrant phenotype. It may also indicate that c-Myb is a positively regulated target gene of v-ErbA, a possibility that is presently pursued further.

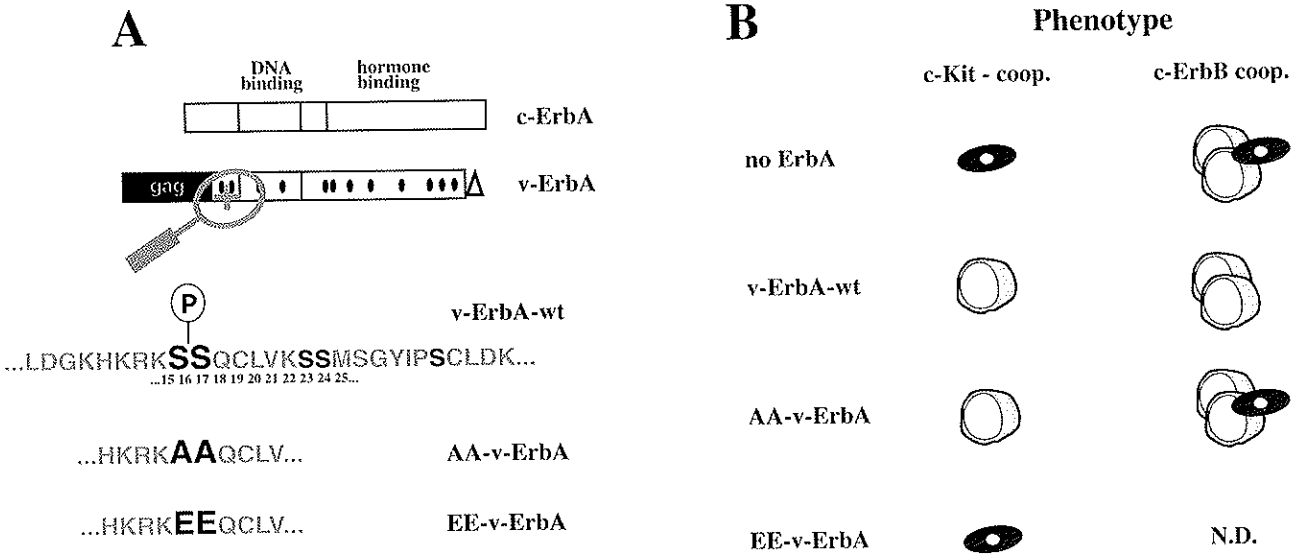

Fig. 5: V-ErbA phosphorylation mutants and their bioactivity in cooperation with c-Kit and c-ErbB
A. *Mutant structure.* The mutants used to test if v-ErbA phosphorylation is important for SCF-induced proliferation and differentiation arrest in normal, c-Kit expressing erythroid progenitors are shown. Serines 16/17 were either changed into unphosphorylatable alanines (AA-v-ErbA) or into glutamic acid residues (EE-v-ErbA), structurally mimicking serine phosphorylation.
B. *Bioactivity of the v-ErbA phosphorylation mutants in erythroblasts: Cooperation with c-Kit and c-ErbB.* SCF/TGFα progenitors expressing v-ErbA, AA-ErbA, EE-ErbA or no ErbA were cultivated in Epo/Ins plus the c-Kit ligand SCF (left) or the c-ErbB ligand TGFα (right). Unexpectedly, AA-v-ErbA cooperated with c-Kit like v-ErbA wt, causing a complete arrest of differentiation (erythroblast symbol). Even more surprising, the supposed constitutively active v-ErbA mutant EE-v-ErbA represented a dead v-ErbA (erythrocyte symbol). However, in cooperation with c-ErbB (right, c-ErbB partially arrests erythroid differentiation on its own, erythroblast plus erythrocyte symbols), AA-v-ErbA lacked biological activity, while v-ErbA-wt showed the expected complete differentiation arrest. N.D., not done. Thus, c-Kit either regulates v-ErbA activity via other putative phosphorylation sites (small black serine residues in A) or v-ErbA phosphorylation plays no role in cooperation with c-Kit.

V-ErbA phosphorylation mutants

The activity of the v-ErbA oncoprotein is crucially dependent on the phosphorylation of two N-terminal serine residues, Ser-16/17, when cooperating with the oncogenic tyrosine kinases ts-v-ErbB and ts-v-Sea. This was shown both by mutating these sites to alanines and by suppressing their phosphorylation via a serine kinase inhibitor. To test in normal erythroid progenitors whether direct phosphorylation of Ser-16/17 was responsible for the c-Kit-regulated activity of v-ErbA, v-ErbA mutants were employed in which Ser 16/17 were either altered to alanine (AA-ErbA) or to glutaminic acid residues (EE-ErbA, **Fig. 5**). The AA-v-ErbA protein was expected to be inactive, while the EE-ErbA protein should be constitutively active, mimicking a constitutively phosphorylated v-ErbA.

The actual behavior of both v-ErbA mutants in the c-Kit-expressing erythroblasts was totally unexpected. Surprisingly, the AA-ErbA protein behaved exactly like wild-type v-ErbA in c-Kit-expressing progenitors, both with respect to self renewal induction and to differentiation arrest. In contrast, the EE-ErbA

seemed to be toxic for the cells, supporting only delayed outgrowth of a small fraction of the infected cells. In this subpopulation, however, EE-v-erbA was totally unable to cause a differentiation arrest in the presence of SCF (**Fig. 5**).

These results showed that phosphorylation of v-ErbA at Ser 16/17 was not required for cooperation with c-Kit. Thus, either phosphorylation is not required for cooperation with c-Kit or v-ErbA has to be phosphorylated at other, unknown sites. When allowed to cooperate with endogenous c-ErbB, however, the AA-ErbA was completely inactive, while v-ErbA showed the expected activity (**Fig. 5**). These results suggest that c-ErbB and c-Kit utilize distinct pathways to cooperate with v-ErbA.

7. Reconstructing murine Friend erythroleukemia in avian erythroblasts

Oliver Wessely and Hartmut Beug,
with C. Tran-Quang and J. Ghysdael (Paris)

In 1994, we were able to determine in chicken erythroblasts the biological activity of two of the three Friend virus oncoproteins, Spi-1 and mutant p53. Spi-1 exhibited a marked ability to arrest erythroid differentiation, particularly when able to cooperate with the residual kinase activity of a biologically inactive v-Sea oncogene. In contrast, mutant p53 alone was unable to arrest differentiation in erythroblasts carrying a conditionally active ts-v-Sea oncoprotein. However, mutant p53 did profoundly alter the growth requirements of normal erythroid progenitors: instead of requiring SCF and TGFα plus estradiol, the mutant p53 expressing cells grew in estradiol only (Tran Quang *et al.*, 1995).

In 1995, we have started to analyze the cooperation of the three Friend virus "onco"proteins, using retroviruses expressing Spi-1, a temperature-sensitive mutant of p53, exhibiting mutant conformation at 39°C and two versions of the murine EpoR, the wild type receptor and a receptor which had been rendered constitutively active by a mutation in the ligand binding domain. It rapidly turned out that double or triple infections of bone marrow with retroviruses expressing one "onco"protein each were too inefficient to yield healthy, well expressing erythroblast clones in sufficient numbers.

To overcome these problems, we developed a new strategy to express two or even three of the Friend virus "onco"proteins in chicken erythroblasts. Two of the three genes (e.g. spi-1 and p53 or spi-1 and EpoR) were cloned into chicken retrovirus vectors connected by an internal ribosomal entry site (IRES) derived from Influenza/Cytomegalo-viruses. These IRES sequences allow the simultaneous translation of several genes from one polycistronic messenger RNA. Therefore, a retrovirus containing two genes of interest connected by an IRES site will express both respective proteins at similar levels. To introduce the third gene, its c-DNA was cloned into a nondefective avian retrovirus vector (RCAS, derived from the Rous sarcoma virus) in which the gene to be expressed was exchanged with the src-gene. Stable transfection of avian cells with defective retroviral DNA requires the simultaneous transfection with nondefective helper virus DNA. Therefore, transfection of fibroblasts with e.g. a defective Spi-1-IRES-p53 vector carrying the neomycin resistance marker plus a nondefective RCAS-EpoR vector should result in fibroblasts expressing all three proteins after suitable G418 selection. Upon cocultivation with bone marrow, both types of virus particles produced (e.g. defective Spi-IRES-p53 particles and nondefective RCAS-EpoR particles) should easily infect the same target cells.

To date, all the required retroviral vectors have been constructed, introduced in various combinations into fibroblasts and shown to express the desired proteins. Testing of the various combinations of Friend "onco"proteins in both ts-v-Sea erythroblasts and normal erythroid progenitors is still in progress. Because of space considerations and to avoid confusion, we decided to give a full report on the final results in 1996 rather than describing the still incomplete findings in this report.

8. The v-ski oncogene cooperates with c-kit to transform normal chick bone marrow cells.

Hartmut Beug and Peter Steinlein,
with R. Dahl and M.J. Hayman (New York)

Last year, we demonstrated that the oncogenic transcription factor v-Ski, together with the ligand activated receptor tyrosine kinase c-Kit, induces the continuous *in vitro* self renewal of primary avian multipotent progenitors, able to differentiate into macrophages, neutrophils, mast-cell like cells and erythroid cells. The multipotent nature of these progenitors was directly demonstrated by limiting dilution cloning, yielding large, immature or immature plus mixed mature cell clones which could be grown into mass cultures and induced to differentiate along the different lineages. Recloning of the multipotent clones again yielded multipotent subclones.

In 1995, we have characterized these multipotent progenitor clones in more detail. We could show that they required the continuous presence of SCF and tested batches of chicken serum for continuous proliferation. Replacement of estradiol by its antagonist ICI 164384 led to a gradual loss of the cell's ability to commit and differentiate into erythroid cells. We were able to replace the chicken serum by a complex mixture of factors, containing estradiol, dexamethasone, SCF, TGFα, chicken myelomonocytic growth factor (cMGF, probably an avian Il-6 homologue) and IGF-1. Under these conditions, the cells could be maintained in chicken serum free medium for prolonged time periods. We currently determine if and how the individual factors contribute to self renewal or commitment ability of the multipotent cells.

Interestingly, the multipotent progenitors induced by ski and SCF have a much longer *in vitro* life span than any other transformed avian hematopoietic cell. While the latter invariably undergo cellular senescence followed by apoptosis after 25-50 generations, various populations of ski-transformed multipotent progenitors exhibited an *in vitro* life span of 90 to >110 generations. However, after this prolonged life span, all cultures underwent typical senescence and no spontaneous immortalization was observed. It is well possible that this altered life span is a characteristic of very early, multipotent hematopoietic progenitors.

We also had a closer look at the ability of these cells to commit and differentiate along the various lineages and at the factors/hormones required for these processes. If density-purified, multipotent cells were seeded directly into media containing differentiation factors (e.g. Epo plus insulin or cMGF/other myeloid-specific cytokine activities) many of the cells died, while the remaining ones differentiated simultaneously into erythroid and myeloid cells, the relative frequencies being a property of the particular clone used. We were however able to devise a strategy by which the cells could be "directed" to commit and differentiate along particular lineages. Clonal strains of multipotent progenitors were first exposed to specific mixtures of growth factors and hormones still containing SCF (e.g. SCF, TGFα, Estradiol, Dex for erythroid cells or SCF, cMGF, ICI 164384, Dex for myeloid cells). Under these conditions the cells divided with identical speed, but gradually altered their phenotype as determined by histological/cytochemical staining and analysis of cell surface antigens by fluorescent antibodies. According to these criteria, most, if not all cells developed into cells committed to either the erythroid or myeloid lineages. These committed cells underwent efficient terminal differentiation when they were treated with the relevant lineage-specific growth/differentiation factors, but underwent apoptosis when exposed to the incorrect factors for the respective lineage. Presently, we try to characterize multipotent or committed cells in more detail for surface marker expression, enabling us to sort single cells into media containing different factor/hormone combinations. This should allow to determine if commitment is a stochastic process or if receptor tyrosine kinase ligands, cytokines and steroid hormones cooperate to determine the fate of a multipotent cell during commitment. Furthermore, we may learn whether commitment is irreversible or reversible.

We also analyzed the multipotent cells, the cells committed to the erythroid or to myeloid lineages and the terminally differentiated cells for their expression of erythroid (globin, GATA-1) and myeloid (mim-1) marker proteins by Western Blot analysis. The cells committed to both the erythroid and to myeloid lineages still coexpressed marker proteins from different lineages, i.e. GATA-1 and mim-1. Only upon terminal differentiation, mim-1 expression was extinguished in the erythroid cells, while GATA-1 was strongly upregulated. Conversely, GATA-1 expression was only abolished in the myeloid-committed cells when these were induced to terminal differentiation into macrophages and mast cells (Beug *et al.*, 1995).

Our results indicate that a combination of v-Ski and activated c-Kit induces long-term self renewal in primary multipotent progenitors, which can be induced to commit and differentiate along specific lineages under different, defined conditions. Our data also suggest that growth factors and steroid hormones control terminal differentiation by a combined induction of commitment, growth and apoptosis, processes which are likely to be affected in stem cell leukemias.

B. Action and cooperation of signal transducer oncoproteins in mammary carcinogenesis

The second major project of our laboratory is to characterize the alterations caused in polarized mammary epithelial cells by single oncoproteins. We chose to study oncoproteins which are involved in signal transduction from growth factor receptors at the cell surface to nuclear factors regulating gene transcription. Our long-term aim is to gain insight into related mechanisms operative in human mammary carcinomas, which frequently contain overexpressed c-Ras and/or overexpressed and/or mutated receptor tyrosine kinases of the EGF receptor family (HER-1, HER-2, HER 4).

1. Reversible loss of polarity in epithelial cells expressing a hormone-inducible c-JunER fusion protein

Irene Fialka, in collaboration with H. Schwarz (Tübingen)

This project, described in detail last year, could be finished in 1995. Last year, we reported that a fusion protein between c-Jun and the hormone binding domain of the estrogen receptor (c-JunER) caused the reversible breakdown of epithelial polarity, when activated by estradiol, both on permeable supports and in collagen gels. Reversible loss of polarity could be demonstrated by redistribution of apical and basolateral markers to the entire cell surface. Molecular changes accompanying the loss of epithelial polarity were also detected. Complex formation between E cadherin and other proteins (β-catenin) in the adherens junctions was weakened. Also, c-JunEr was found to up- or downregulate other members of the Jun family (JunB, JunD) as well as several known AP-1 target genes.

In 1995 we addressed one still open question, i.e. whether or not the effect of c-JunER could be related to that of endogenous c-Jun. Since c-Jun requires activation by upstream signal transduction, we expected that c-JunER would be insensitive to activation by estradiol, if the cells had been previously deprived of growth factors. This was exactly the result obtained. The hollow, tubular structures obtained in collagen gels plus growth factors but without estradiol failed to collapse upon estradiol addition, if the respective collagen gel was switched to serum-free medium lacking growth factors one day before addition of E2. In line with these findings, the tubular structures obtained from control EpH4 cells that did not express any JunER protein could be induced to collapse after addition of acidic FGF, a growth factor known to signal via c-Jun (Fialka *et al.*, 1996).

2. Ha-Ras cooperates with TGFβ1 to induce epithelial/fibroblastoid conversion and invasiveness in polarized mammary epithelial cells

Martin Oft, in collaboration with E. Reichmann and J. Peli (Lausanne)

Last year, we concentrated on a new model of mammary carcinogenesis, i.e. Ha-Ras transformed, mouse mammary epithelial cells that form rapidly growing tumors upon injection into syngeneic mice. We demonstrated that this tumor forming ability is due to a highly plastic epithelial cell phenotype, both *in vivo* or by culture in serum-free collagen gel matrices. Polarized epithelial Ep-ras cells undergo epithelial-fibroblastoid conversion (EFC) during tumor formation in mice, a process which can be reproduced *in vitro* by cultivating the cells in serum containing collagen gels. In the absence of serum, the same Ha-Ras transformed cells remained polarized, similar to normal mammary epithelial cells which were unable to undergo such EFC under any of these conditions, both *in vivo* and *in vitro*.

In 1995, we tried to identify the factor(s) responsible for this serum-induced EFC. Transforming growth factor (TGF) β1 turned out to be the major factor that regulates EFC. In serum-free collagen gels, TGF-β1 induced EFC in Ras-transformed, but not normal cells. Ras cells grown in collagen gels in the absence of TGF-β1 retained a polarized phenotype, including basolateral expression of several respective markers such as E-cadherin, β-catenin and ZO-1. After induction of EFC, *in vitro* by TGF-β1 or *in vivo,* the cells exhibited many fibroblastoid characteristics such as loss of E cadherin- and ZO-1 expression and gain of vimentin expression. Interestingly, after EFC the cells also produced and deposited TGF-β1, while the parental, epithelial Ha-Ras cells failed to produce TGF-β1, similar to normal control cells.

When the fibroblastoid cells obtained after EFC or from tumors were cultivated at very low, clonal densities, they gradually reverted to a polarized, epithelial phenotype. This suggested that TGF-β1 could be involved in maintaining the fibroblastoid phenotype obtained after EFC via an autocrine loop.

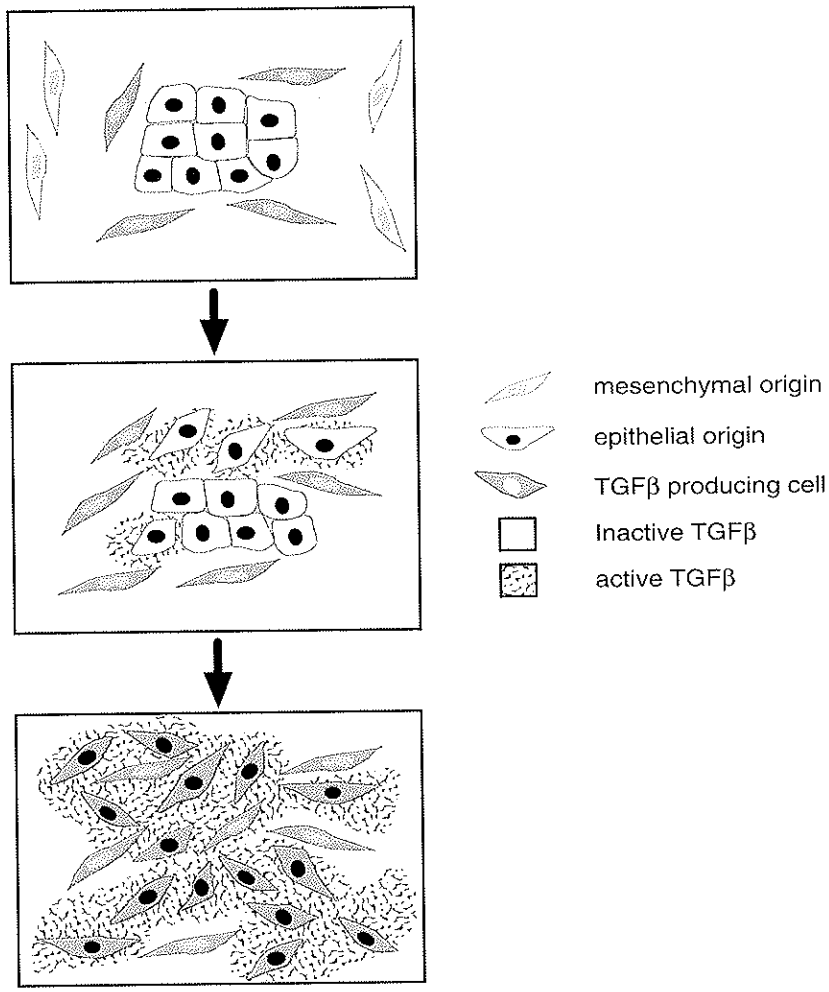

Fig. 6: Model for TGFβ1 action in tumor development.
In this simple scheme, we postulate that TGFβ1 (gray CCC area) is initially provided by cells surrounding and infiltrating the tumor, such as fibrocytes, endothelial cells, lymphocytes and macrophages (top panel, cells with dotted nuclei). The interaction of these different, mostly mesenchymal cell types with the (epithelial) tumor cells (black nuclei) may trigger the efficient activation of TGFβ1 (middle panel). The secreted and activated TGFβ1 (black CCC areas) in turn may cause certain epithelial tumor cells to undergo EFC, i.e. to convert to a fibroblastoid and invasive phenotype. The converted fibroblastoid cells begin to produce TGFβ1 themselves (bottom panel, cells with gray cytoplasm), thus generating an autocrine loop which facilitates the maintenance of the fibroblastoid phenotype and the recruitment of additional cells into EFC and invasiveness.

This notion could be confirmed by a neutralizing antibody to TGF-β1, which rapidly converted the fibroblastoid cells into epithelial ones. The same antibody also neutralized the activity in serum that induced EFC. This led to the concept that TGF-β1 has two important effects on Ha-Ras epithelial cells, which it fails to evoke in normal control cells: (i) induction of EFC and (ii) induction of TGF-β1 production in the converted cells, thus maintaining EFC via an autocrine TGF-β1 loop.

This concept could at least in part be shown to apply *in vivo* (see **Fig. 6**). First, young tumors in which the cells were still epithelial failed to express TGF-β1 in the tumor itself, but evoked TGF-β1 production in the stromal fibroblasts surrounding the tumor (**Fig. 6**). Older, fully established tumors, in which the cells of donor origin had become vimentin positive and which therefore most likely had undergone EFC, did express TGF-β1 in the tumor itself (**Fig. 6**).

More importantly, we could also show that exogenous TGF-β1 could promote EFC *in vivo*: Mice were injected with Ras-transformed epithelial cells, next to an implanted slow-release bead, which was loaded with TGF-β1 or, in the control, with BSA. While the cells in the control mice remained epithelial after 4 days and formed a tumor only after 20 days, the cells injected next to the TGF-β1-bead underwent EFC within 4 days and formed a tumor much more rapidly. These results indicate (i) that Ras-transformed mouse epithelial cells induce and/or activate TGF-β1 in the surrounding stroma and (ii) that this activated TGF-β1 is directly responsible for EFC during tumor formation *in vivo*.

Since the TGFβ family of growth factors is highly involved in morphogenetic processes during embryonic development we also re-investigated the role of

TGFβ1 on normal mammary gland development. *In vitro* organogenesis in collagen gels was totally abrogated in the complete absence of TGFβ1, but strongly stimulated by low concentrations (0.1 ng/ml) of the factor. At higher concentrations of TGFβ1 (> 0.25 ng/ml) which are required to induce EFC in the Ep-Ras cells, normal epithelial cells underwent apoptosis. In analogy to these *in vitro* findings, we could also show that during endbud outgrowth of the normal mammary gland TGFβ1 is produced exclusively in the mesenchymal stroma surrounding the growing epithelial structures. During involution of the mammary gland after weaning, a process that involves massive apoptosis *in vivo*, TGFβ1 is only expressed in the mesenchyme surrounding the apoptotic alveoli, again suggesting an epithelial induction of mesenchymal TGFβ1 production.

Taken together, our results suggest that activated Ras (or overexpressed receptor tyrosine kinases activating the Ras pathway) cooperate with theTGF-β1 receptor in both normal development and carcinogenesis. This seems to involve processes like induction/activation of stromal TGFβ1 by interaction of epithelial and mesenchymal cells and epithelial-fibroblastoid conversion induced and maintained by TGFβ1. The main difference between normal and tumor cells thus seems to be that normal regulation of these processes is disrupted in the tumor cell (M. Oft *et al.*, manuscript submitted).

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Transcription factors involved in early development, differentiation and oncogenesis

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Technician	Paula GRANINGER

Introduction

Early development and differentiation processes depend on differential gene expression which is brought about by the interplay of tissue-specific and ubiquitous transcription factors. We are interested in the role of the **pa**ired bo**x**-containing (*Pax*) transcription factors in these biological processes. The *Pax* genes constitute a family of developmental control genes which were initially identified in *Drosophila* (reviewed by Noll, 1993). Nine members of the vertebrate **pa**ired bo**x**-containing (*Pax*) gene family have since been isolated by homology to these *Drosophila* genes. Their distinct spatiotemporal expression pattern in the vertebrate embryo has implicated these genes in the control of morphogenesis and pattern formation (reviewed by Stuart *et al.*, 1994). Indeed, mutations in *Pax-1*, *Pax-3* and *Pax-6* have been associated with the mouse developmental mutants *undulated*, *Splotch* and *Small eye*, respectively. In humans, genetic lesions in the *PAX-3* gene are known to generate Waardenburg's syndrome, while mutations in the *PAX-6* gene cause the disorders aniridia and Peters' anomaly. *PAX-3* and *PAX-7* have furthermore been implicated in the genesis of alveolar rhabdomyosarcomas, as these myogenic tumors are characterized by specific chromosome translocations resulting in the fusion of one of the two *PAX* genes to a gene of the fork head transcription factor family. A potential role of *Pax* genes in oncogenesis has been further suggested by ectopic expression experiments in fibroblasts (reviewed by Stuart *et al.*, 1994).

Previously, we have studied in detail the *PAX-5* gene which codes for the transcription factor BSAP (reviewed by Busslinger and Urbánek, 1995). BSAP binds to its target genes via its N-terminal paired domain and uses a C-terminal module of activating and inhibitory sequences to regulate gene transcription (see **Fig. 1**). BSAP is expressed at all stages of B-cell development except in terminally differentiated plasma cells and is known to regulate the *CD19* gene as well as the *Iε* promoter and 3'α enhancer of the immunoglobulin heavy-chain gene locus (reviewed by Busslinger and Urbánek, 1995). In addition to all B-lymphoid tissues, the *Pax-5* gene is also expressed in the embryonic midbrain and adult testis of the mouse (Adams *et al.*, 1992). Consistent with this expression pattern, gene inactivation in the mouse germline demonstrated that *Pax-5* plays an essential role in B-lymphopoiesis and midbrain development (Urbánek *et al.*, 1994). *PAX-5* has also been implicated in oncogenesis, as deregulated expression of this gene was consistently observed in medulloblastoma, the most common brain tumor of childhood (Kozmik *et al.*,1995) and in malignant astrocytoma (Stuart *et al.*, 1995).

During the last year we have discovered and investigated novel aspects of the *Pax-5* mutation which affect B-cell and midbrain development. We have furthermore initiated experiments to identify regulatory factors that act upstream of *Pax-5* in the hierarchy of transcriptional control during CNS and B-lymphocyte development. In addition, characterization of the *PAX-5* locus revealed that this gene is transcribed from alternative promoters in its three expression domains. Interestingly, analysis of these promoter sequences led to the observation that the *PAX-5* gene is deregulated by a recurring t(9;14)(p13;q32) chromosomal translocation in a specific subset of human B-cell lymphomas.

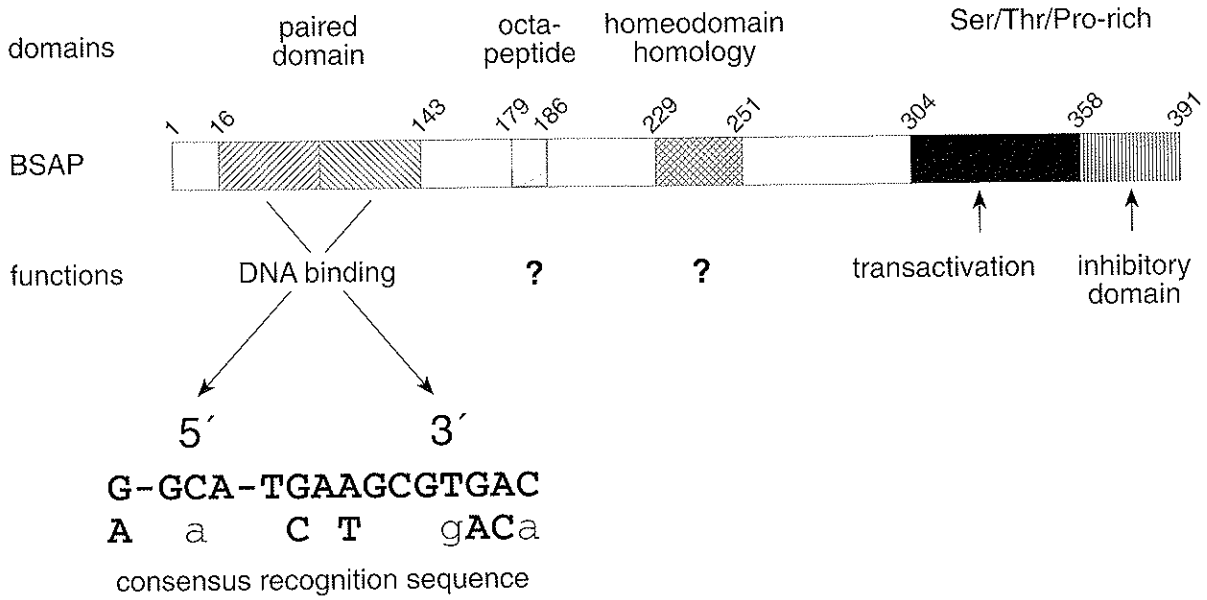

Fig. 1: Structure of the BSAP (Pax-5) and its consensus recognition sequence.
A schematic diagram of the different domains of BSAP is shown together with the corresponding amino acid positions. The consensus recognition sequence has been recently up-dated (Czerny and Busslinger, 1995), and the C-terminal regulatory module consisting of activating and inhibitory sequences was described by Dörfler and Busslinger (1996).

Pax-5 is required for B-lymphoid commitment in fetal life and for progression beyond an early pro-B cell stage in adult B-lymphopoiesis.

Stephen L. Nutt and Pavel Urbánek in collaboration with Antonius Rolink (Basel Institute for Immunology)

B-cell development involves the successive differentiation of pluripotent stem cell through different stages characterized by the progenitor (pro-B), precursor (pre-B) and immature B-cells which, upon antigen stimulation, differentiate into immunoglobulin-secreting plasma cells. These developmental stages are defined by the expression of stage-specific cell surface molecules and by the status of rearrangements of the immunoglobulin heavy (IgH) and light (IgL) chain gene loci.

The B-cell-specific transcription factor BSAP which is encoded by the *Pax-5* gene is expressed throughout B-lymphopoiesis except in terminally differentiated plasma cells. We have previously shown that targeted disruption of the murine *Pax-5* gene leads to a complete block in B-cell development at an early age (Urbánek *et al.*, 1994; see **Fig. 2A**). To identify the mechanism of this developmental block as well as to further elucidate the role of BSAP in B-lymphopoiesis we have continued the analysis of the Pax-5 (-/-) phenotype *in vivo* and *in vitro*.

Flow cytometric analysis of bone marrow of *Pax-5* mutant mice revealed large cycling cells displaying a surface phenotype characteristic of pro-B cells ($B220^+$, $CD43^+$, c-Kit^+, IL-$7R^+$, BP-1^-, IL-$2R^-$, $sIgM^-$). Single cell PCR analysis of the IgH and IgL_κ genes demonstrated that the majority of these cells have undergone D_H-J_H rearrangements. Although both RAG genes are normally expressed in Pax-5 (-/-) pro-B cells, not a single V_H-D_H-J_H or V_κ-J_κ rearrangement could be identified, indicating that *Pax-5* is required to complete immunoglobulin gene rearrangement. Pro-B cells from *Pax-5* (-/-) bone marrow could be cultured *in vitro* on stromal cells in the presence of IL-7 with the same frequency as wild-type cells. Long-term cultures of these cells displayed a cell surface phenotype comparable with that obtained *in vivo*.

The expression of a number of B-cell-specific genes was analyzed in cultured *Pax-5* (-/-) pro-B cells by RNase protection analysis, which indicated that genes coding for known B-cell-specific transcription factors were expressed at wild-type levels. Several B-lymphoid genes (*CD19*, *blk*, *λ5* and *VpreB*) have been suggested to be regulated by BSAP (Pax-5) (reviewed by Busslinger and Urbánek, 1995). However, only *CD19* expression was shown to be affected by the absence of BSAP, thus providing genetic evidence that *CD19* is a BSAP target gene. However, B-cell development proceeds normally in CD19-deficient mice (Rickert *et al.*, 1995), and hence *CD19* is not one of the critical target genes respon-

sible for the early differentiation block observed in the bone marrow of *Pax-5* (-/-) mice. To initiate a systematic search for such target genes, we have generated an estrogen-regulable BSAP-ER protein by fusing the hormone-binding domain of the human estrogen receptor (ER) to the C-terminus of BSAP. *Pax-5* (-/-) pro-B cells infected with a BSAP-ER expressing retrovirus rapidly induce *CD19* expression and differentiate *in vitro* to sIgM-positive cells in response to estrogen treatment. These BSAP-ER expressing pro-B cells will be used to identify critical BSAP target genes by subtractive cDNA cloning techniques. B-lymphopoiesis in the fetal liver predominantly generates pro-B cells which can be readily cloned and cultured from wild-type and heterozygous embryos. In contrast, no pro-B cells could be obtained from *Pax-5* (-/-) fetuses. This finding was further supported by the observation that no pro-B cells could be detected by flow cytometric analysis in the fetal liver of *Pax-5* (-/-) embryos. These results point to a difference in the dependency of fetal and adult B-lymphopoiesis on BSAP function. In the adult mouse, BSAP (Pax-5) is critical for the transition from the pro-B to pre-B stage of B-cell development, while in the fetal liver BSAP is already required for the commitment of hematopoietic progenitor cells to the B-lymphoid lineage (see **Fig. 2B**).

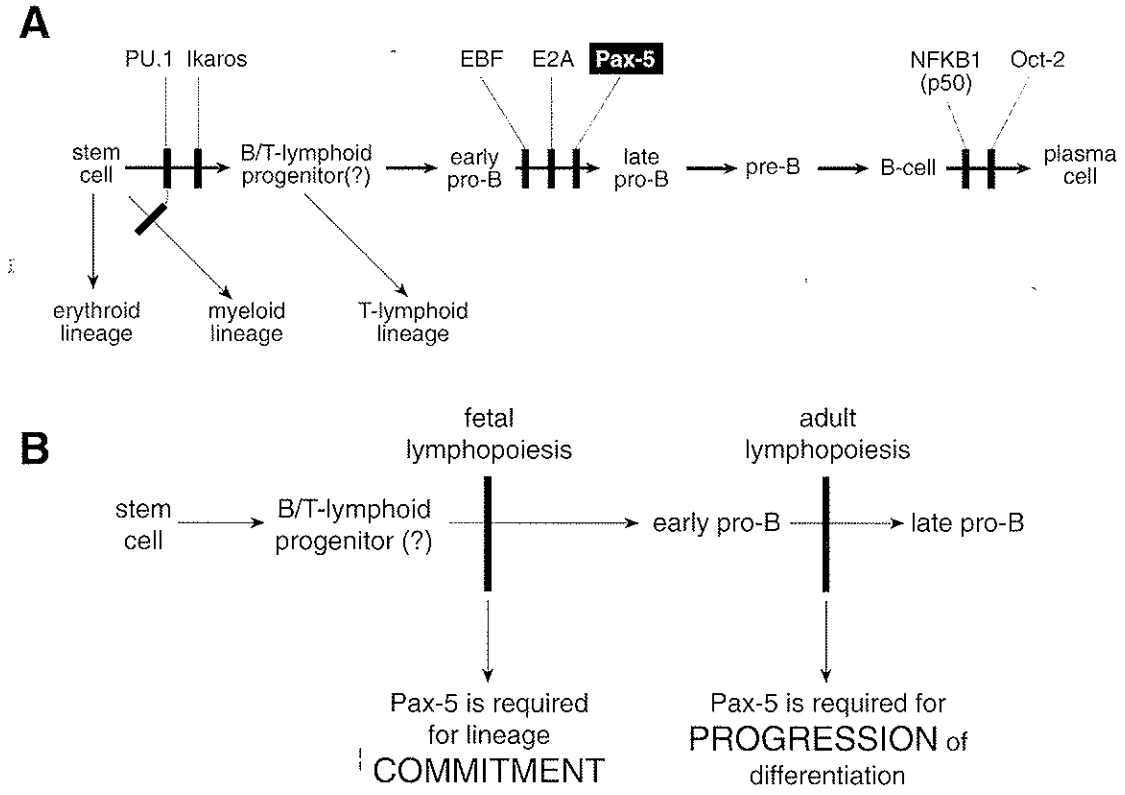

Fig. 2: Hierarchy of transcriptional control of B-cell development.
A) A schematic diagram of B-cell development is shown together with the developmental block observed in the bone marrow of mice that are homozygous for targeted mutation in one of the indicated transcription factor genes. For further explanation, see Busslinger and Urbánek (1995) . **B**) Differential dependency of fetal and adult B-lymphopoiesis on Pax-5 function.

Pax-5 and Pax-2 regulate midbrain and cerebellum development in a dosage-dependent manner

Pavel Urbánek in collaboration with Ingrid Fetka and Miriam H. Meisler (University of Michigan)

Pax-5 and *Pax-2*, two closely related members of the vertebrate *Pax* gene family, are expressed with a similar spatial and temporal pattern in the developing CNS, particularly in the region of the midbrain-hindbrain boundary (Rowitch and Mc Mahon, 1995). We have previously shown that targeted disruption of the mouse *Pax-5* gene leads to morphological alterations within this brain region (Urbánek *et al.*, 1994). Recently, a transgene-induced deletion of a chromosome 19 segment, including the *Pax-2* locus, has been reported in the *Krd* mice (Keller *et al.*, 1994; see also **Fig. 3A**).

Although this semidominant mutation causes kidney and retinal defects in *Krd* hemizygous animals (consistent with *Pax-2* expression in these organs), it appears to have no gross effect on brain morphogenesis. To investigate a possible interaction between the *Pax-5* and *Pax-2* genes during CNS development, we have generated *Pax-5/Krd(Pax-2)* double mutant mice. Most of *Pax-5* (+/-)/*Krd* (+/-) animals show a phenotype similar to that of *Pax-5* (-/-) mice, i.e. the most posterior part of the midbrain consisting of the inferior colliculi is underdeveloped in the midline. However, in 25% of animals from this group, the structure of the middle part of the cerebellum, the so-called vermis, is completely disrupted, apparently due to a failure of cerebellar primordia to fuse during development. Although these mice suffer from impaired motor control resulting in swaying, they reach adulthood and are fertile. In contrast, newborn *Pax-5* (-/-)/*Krd* (+/-) mice lack the posterior midbrain and most of the cerebellum (see **Fig. 3B**), and die shortly after birth.

These data suggest that *Pax-5* and *Pax-2* together specify morphogenesis of the posterior midbrain and cerebellum in an interactive and dosage-dependent manner. We speculate that these genes are required for controlling the proliferation of localized neuroectodermal progenitor cell populations during CNS development. Experiments are under way to test this hypothesis and to study the role of Pax-2 and Pax-5 in midbrain-specific gene regulation by analyzing the expression of putative target genes in *Pax-5* (-/-)/*Krd* (+/-) embryos.

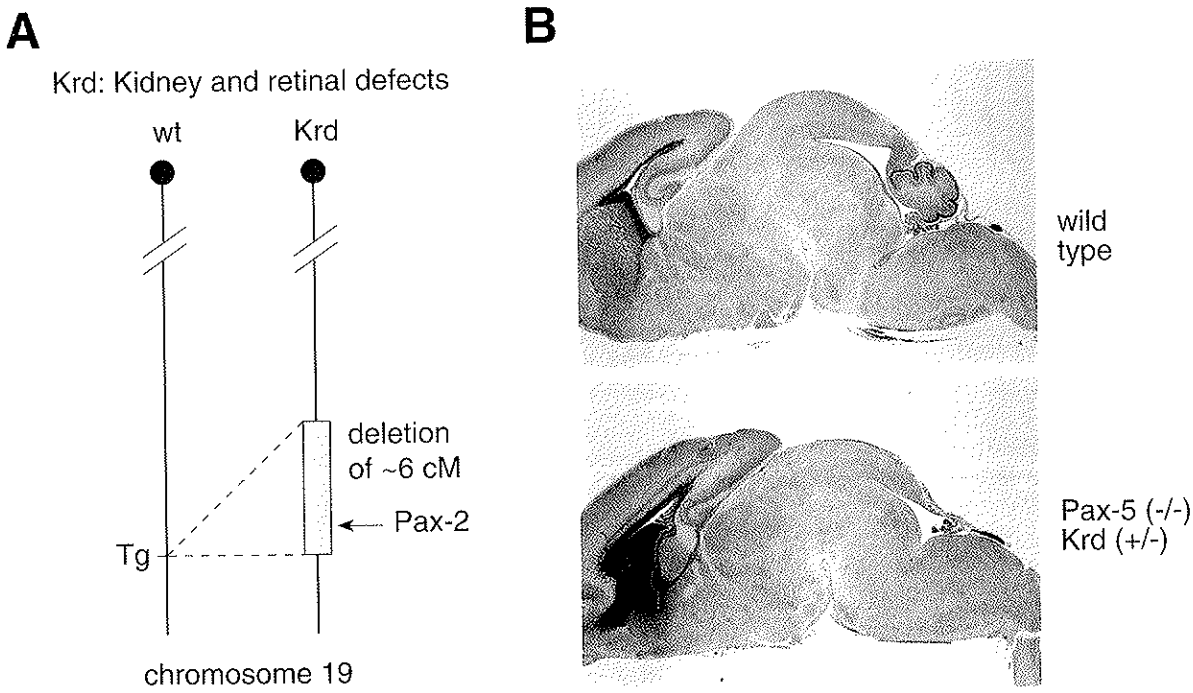

Fig. 3: Brain phenotype of *Pax-5/Krd(Pax-2)* double mutant mice.
A) Schematic diagram of the *Krd* deletion on mouse chromosome 19. *Krd* stands for **K**idney and **r**etinal **d**efects (Keller *et al.*, 1994).
B) Midsagittal sections through the brain of a newborn wild-type and *Pax-5* (-/-)/*Krd* (+/-) mutant mouse.

Characterization of the *PAX-5* locus and identification of alternative *PAX-5* promoters

Norman Klix, Zbynek Kozmik and Peter Pfeffer

We have isolated the entire human *PAX-5* locus and the 5' region of the mouse *Pax-5* gene on overlapping YAC, P1 and cosmid clones, indicating that this locus is at least 100 kb in size. The human and mouse *Pax-5* genes are both transcribed from two distinct promoters, thus resulting in splicing of two alternative 5' exons (1A and 1B) to the common coding sequences of exons 2-10 (**Fig. 4**). The two promoters are differentially regulated during development, as shown by RNase protection analysis with exon 1-specific riboprobes. Transcription from the upstream promoter initiates at a single site downstream of a TATA box and occurs predominantly in B-lymphocytes. In contrast, the TATA-less downstream promoter results in heterogeneous transcription initiation and is active in all *Pax-5* expression domains.

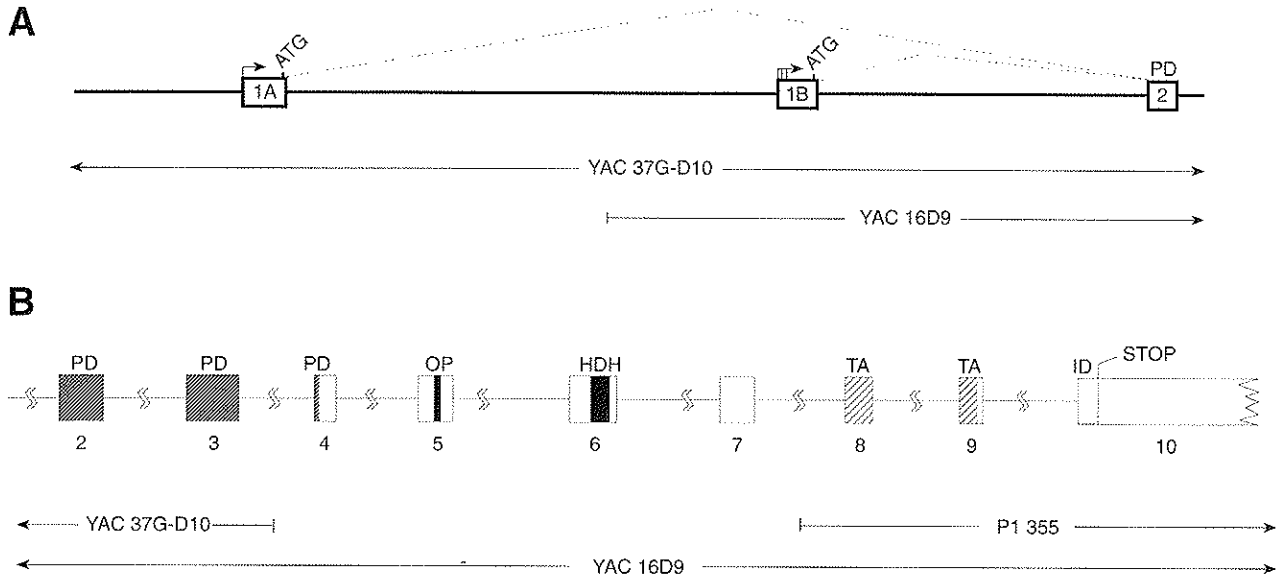

Fig. 4: Structural organization of the *PAX-5* gene.
Map of the 5' region (**A**) and the coding sequences (**B**) of the *PAX-5* gene.The extent of *PAX-5* sequences which were isolated on different YAC and P1 clones is shown below a map of the exon-intron structure of *PAX-5*. Abbreviations: PD, paired domain; OP, conserved octapeptide; HDH, homeodomain homology region; TA, transactivation domain and ID, inhibitory domain. For details, see Busslinger *et al.* (1996) .

Analysis of regulatory regions of the *Pax-5* gene in transgenic mice

Peter L. Pfeffer

Pax-5 is expressed in the developing CNS (at the midbrain-hindbrain junction and in the spinal cord) and in B-lymphoid cells where it plays an essential role. It is therefore one of our long-term aims to identify regulatory factors that act upstream of *Pax-5* in the network controlling midbrain development and B-cell differentiation. To this end we have initiated an analysis of the regulatory regions of the *Pax-5* gene. Two complementary approaches were used to delineate the regulatory sequences responsible for tissue-specific regulation of *Pax-5.* Firstly, we are analyzing the 5' region of the *Pax-5* gene in transgenic mice by using enhancer trap techniques and transgenesis approaches. Secondly, we are subjecting the B-cell-specific promoter to a functional analysis in human B-cell lines using Epstein-Barr virus-derived episomal vectors (see below).

As a starting point for the transgenic analysis of the *Pax-5* regulatory regions we have generated a transgene consisting of 25 kb of genomic sequences 5' of exon 2 that were fused in-frame (in exon 2) to a *lacZ* reporter gene (**Fig. 5**). This genomic region encompasses 14 kb of sequence upstream of exon 1A, both exons 1 and the first two introns of the mouse *Pax-5* gene. Transient transgenic embryos as well as permanent transgenic lines were established by micro-injection of this transgene into mouse egg pronuclei. One out of eight transgenic lines expressed the *lacZ* transgene in the midbrain-hindbrain junction of day-12.5 embryos in a manner similar to heterozygous Pax-5 (+/-) embryos (**Fig. 5**). These data indicate that the 25 kb region analyzed contains most, if not all, information for correct midbrain-specific expression of the *Pax-5* gene. The 25 kb region will next be subdivided into smaller fragments for analysis in the enhancer trap vector pGZ40 (Yee and Rigby, 1993) which ultimately should result in a more precise definition of the relevant regulatory sequences.

Characterization of the B cell-specific promoter of *Pax-5* in stably transfected human cell lines

Susanne Vambrie

The *Pax-5* gene is also expressed in all B-lymphoid tissues. In an attempt to identify regulatory factors acting upstream of *Pax-5* in the hierarchy of hematopoiesis, we have subjected the B cell-specific control region of *Pax-5* to a functional analysis in stably transfected human cell lines. For these experiments we have taken advantage of the fact that plasmids containing the EBNA-1 gene and

oriP sequences of the Epstein-Barr virus replicate as episomes in human cell lines (Yates *et al.*, 1985). Reporter genes present on these EBNA-based vectors (REP3) are consequently not integrated into the host genome, are thus not influenced in their transcription by position effects and can be quantitated by Southern blot analysis to normalize expression in different cell lines for copy number effects.

The B-cell-specific promoter of *Pax-5* contains a TATA-box which is located 30 bp upstream of the transcription initation site (see **Fig. 4**). Different fragments extending from the TATA box up to 12 kb into the 5' flanking region were linked in the B-cell-specific exon 1A to a luciferase gene and cloned into the REP3 vector. These different promoter constructs were transfected into human B-lymphoid, T-lymphoid and epithelial cell lines, and the luciferase activity of resistant cell pools was measured and normalized for cell and copy numbers. These experiments indicated that sequences located between 9 and 12 kb upstream of exon 1A harbour B-cell-specific regulatory element(s). This 3-kb region is currently being subdivided and tested for enhancer function.

DNase I hypersensitive sites in the chromatin are often good indicators of important protein-DNA interactions. We have therefore used unique DNA probes to screen the 5' flanking region of exon 1A for DNase I hypersensitive sites which are specific for B-lymphoid cell lines. These experiments led to the identification of a B-cell-specific hypersensitive region which maps ~10 kb upstream of exon 1A. These results together with the functional data described above strongly suggest that an enhancer in the -10 kb region is responsible for B-cell-specific transcription of the *Pax-5* gene.

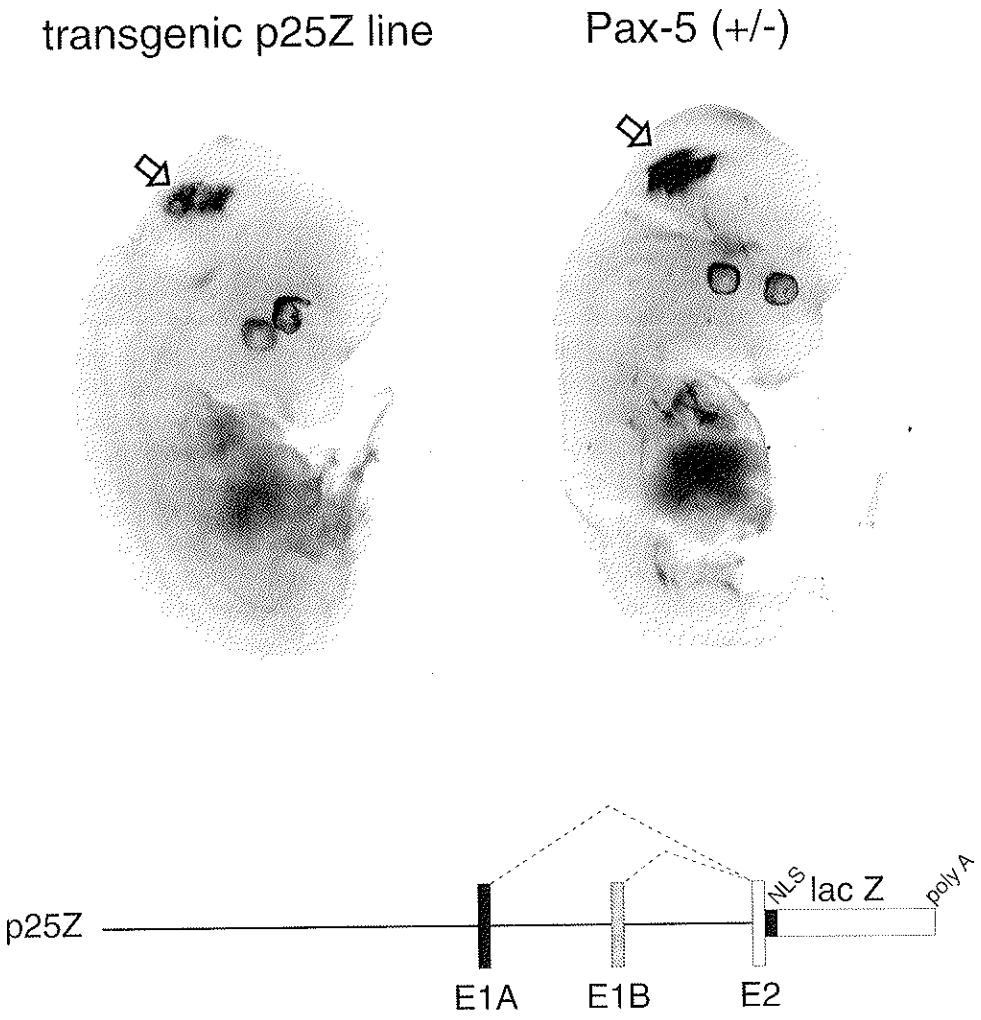

Fig. 5: Identification of a midbrain-specific enhancer in transgenic mice.
LacZ staining of an embryo of the transgenic line p25Z and of a heterozygous *Pax-5* mutant embryo at day 12.5. Arrows point to the mid-hindbrain region expressing the *lacZ* gene. A schematic diagram of the *Pax-5* transgene p25Z is shown in the bottom part.

Deregulation of the *PAX-5* gene by translocation adjacent to the Eμ enhancer of the *IgH* locus defines a subset of B-cell lymphomas.

Zbynek Kozmik, Norman Klix and Paula G. Graninger

We have previously localized the *PAX-5* gene to human chromosome 9p13 (Stapleton *et al.*, 1993). This chromosomal region is involved in recurring t(9;14)(p13;q32) translocations which are highly correlated with small lymphocytic lymphomas of the plasmacytoid subtype (Offit *et al.*, 1992). This subset of B-cell lymphomas is initially of low grade, but displays a tendency to transform into more aggressive large-cell lymphomas. A previous molecular analysis of a t(9;14) breakpoint from a diffuse large-cell lymphoma (KIS-1) demonstrated that the immunoglobulin heavy-chain locus (*IgH*) on 14q32 was juxtaposed to chromosome 9p13 sequences of unknown function (Ohno *et al.*, 1990). By Southern blot analysis and direct sequence comparison we have now mapped the KIS-1 translocation breakpoint 1807 base pairs upstream of exon 1A of *PAX-5*. As a consequence, the potent Eμ enhancer of the immunoglobulin heavy-chain gene is juxtaposed adjacent to the two *PAX-5* promoters (**Fig. 6**). *PAX-5* has been implicated in the proliferation control of mature B-cells (Wakatsuki *et al.*, 1994), and its transcription is known to be switched off upon terminal differentiation to plasma cells. As the Eμ enhancer is highly active in plasma cells, we hypothesize that insertion of this enhancer prevents inactivation of the *PAX-5* locus in B-lymphocytes undergoing terminal differentiation. Our data suggest therefore that deregulation of the *PAX-5* gene by the t(9;14)(p13;q32) translocation contributes to the pathogenesis of small lymphocytic lymphomas with plasmacytoid differentiation.

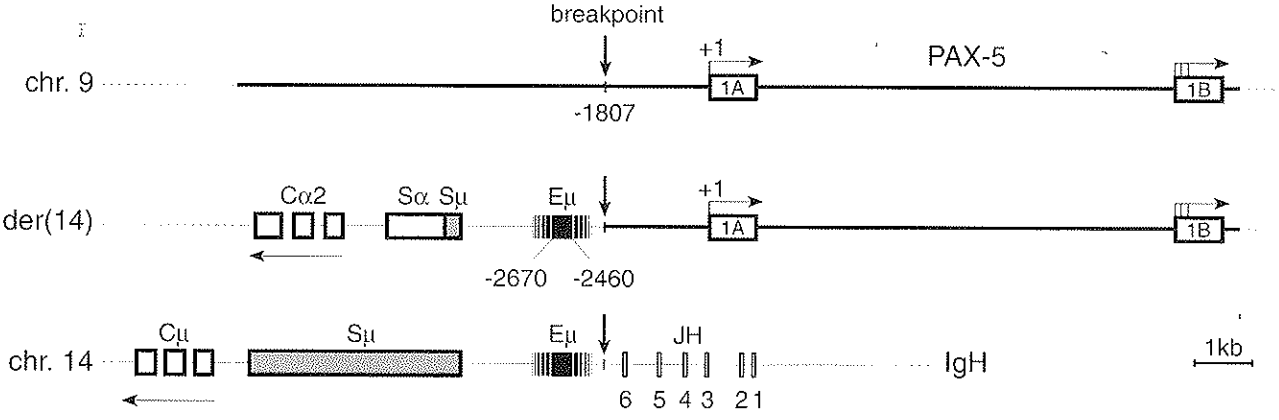

Fig. 6: Juxtaposition of the Eμ enhancer of the *IgH* locus next to the two alternative *PAX-5* promoters in the diffuse large-cell lymphoma KIS-1. Schematic diagram of the 5' region of the *PAX-5* gene, the J_H-to-Cμ region of the immunoglobulin heavy-chain gene (*IgH*) and the corresponding sequences present on the derivative chromosome 14 of KIS-1 cells containing the t(9;14)(p13;q32) translocation (Ohno *et al.*, 1990). The core region of the intronic Eμ enhancer (Rabbitts *et al.*, 1983) is shown together with its nucleotide positions relative to the *PAX-5* transcription start site on derivative chromosome 14.

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The yeast cell cycle

Group leader	Kim NASMYTH
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Postdoc	Thomas BÖHM (until Nov. 95)
Postdoc	Stefan IRNIGER
Postdoc	Simonetta PIATTI
Postdoc	Christine MICHAELIS
Postdoc	Wolfgang ZACHARIAE
Postdoc	Ralf JANSEN
Postdoc	Masaki SHIRAYAMA (since July 95)
Postdoc	Tae Ho SHIN (since Feb. 95)
PhD student	Robert SIEGMUND (until Sept. 95)
PhD student	Christian DAHMANN (until Sept. 95)
PhD student	Dunja KNAPP
PhD student	Isabel GONZALEZ
PhD student	Rafal CIOSK (since Oct. 95)
PhD student (visiting)	Nicoletta BOBOLA
Diploma student	Silke PICHLER (since Dec. 95)
Technical assistant	Marta GALOVA

Introduction

Despite major advances in recent years, our understanding of how cells proliferate and differentiate, let alone how they choose between these two options, remains rudimentary. Four sorts of questions stand out: how do cells coordinate their growth and division, how do cells coordinate the duplication and segregation of their chromosomes, how do external signals influence cells to differentiate or divide, and how do cells sometimes give rise to progeny that behave differently? One of the major advances in cell biology of the past ten years has been the discovery that the architecture of eukaryotic cells and the regulation of their division is highly conserved. This means that fundamental questions can be addressed using organisms whose study is more tractable and the answers gleaned are valuable in directing subsequent human studies. Our current knowledge of cell cycle control has been pioneered by genetic analyses of two distantly related yeasts, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. Such studies have revealed the role of a highly conserved class of protein kinase dependent on regulatory cyclin subunits (the CDK kinases encoded by *CDC28* and *cdc2* genes). Cell cycle dependent fluctuations in different forms of these kinases, distinguished primarily by the type of cyclin subunit, play an important part in the alteration of S and M phases (**Fig. 1**). Activation of the CDK kinase by G1-specific cyclins in late G1 trigger the initiation of DNA replication, whereas activation by G2-specific cyclins in G2 causes entry into mitosis. We have made important progress recently in understanding how the cell cycle is started by transcription of G1 cyclins and S phase proteins, how S phase is regulated by a CDK inhibitor that must be degraded in late G1, how cells prevent re-replication during G2, and how proteolysis of mitotic cyclins is regulated.

Our genetic studies on cyclin proteolysis have led to the identification of a major new player in cell cycle control called the **A**naphase **P**romoting **C**omplex (APC) or Cyclosome. Our studies suggest that the APC is necessary for degrading not only cyclins but also proteins (thus far unidentified) that inhibit the separation of sister chromatids at the metaphase to anaphase transition. The APC promotes cyclin B degradation by causing the ligation of multiple ubiquitin molecules, which marks the cyclin subunit of a cyclin/Cdk complex for degradation by the 26S proteasome (**Fig. 2**). The APC is a large particle with a sedimentation velocity of 20S that contains at least seven different polypeptides. Its regulation during the cell cycle may prove to control several key transitions. One of the more exciting problems for the future is to identify other proteins whose degradation depends on the APC and thereby to evaluate the

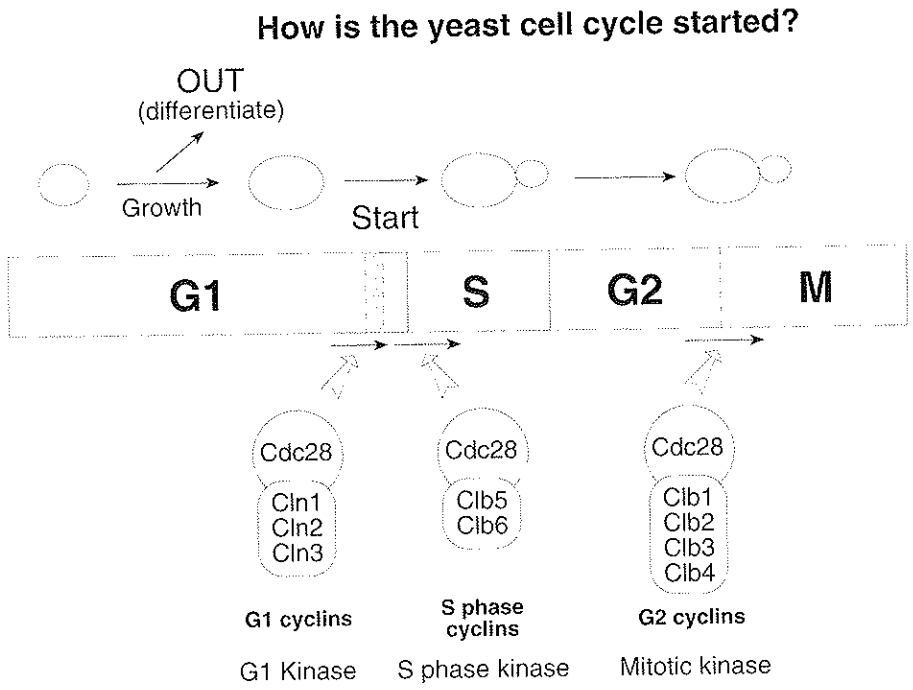

Fig. 1: Cyclin dependent kinases involved in regulating the yeast cell cycle

importance of protein proteolysis in cell cycle control. Thus far our picture of the cell cycle has tended to emphasize the role of Cdks in catalyzing key cell cycle transitions. The identification of the APC promises to add a new dimension to our understanding of regulatory mechanisms at the heart of the cell cycle.

Studies on the budding yeast have also shed important insights into the relationship between proliferation and differentiation. Haploid cells with opposite mating types cause each other to arrest in the G1 phase of the cell cycle and to differentiate into gametes that subsequently conjugate to form diploid zygotes. Cell division in yeast occurs by budding and therefore gives rise to asymmetric progeny: a mother and a daughter cell. Only mother cells express the *HO* endonuclease gene and thereby switch their mating type (**Fig. 3**). This year, we have made major strides in understanding the mechanism by which differential *HO* expression in mother and daughter cells is established. We have identified a repressor, Ash1, whose preferential accumulation in the daughter nucleus at the end of anaphase depends on a set of cytoskeletal proteins including a type V minimyosin called Myo4.

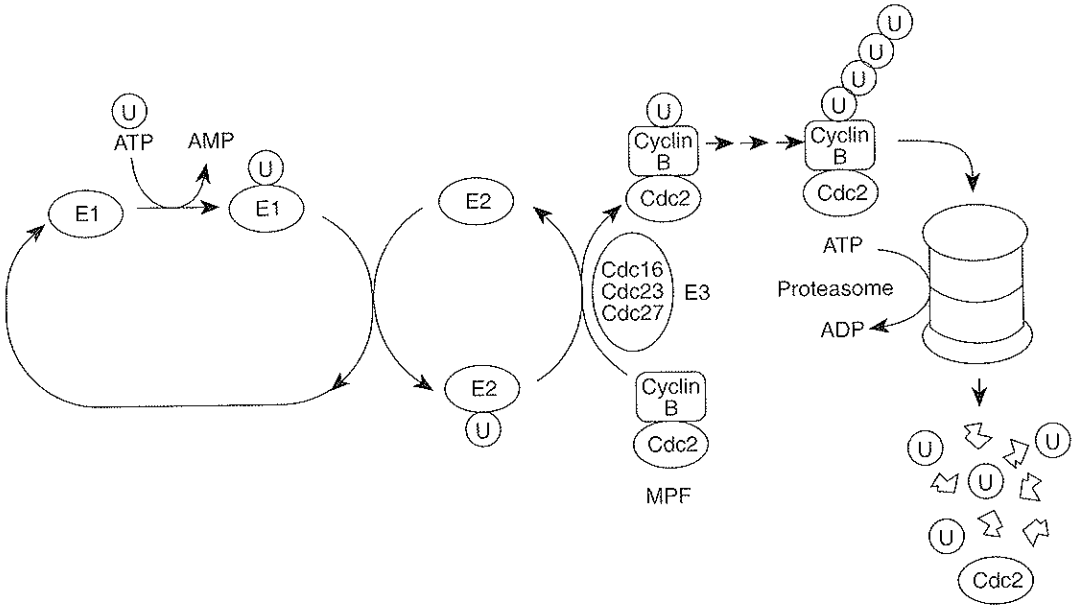

Fig. 2: Ubiquitination mediates cyclin degradation by the proteasome. The ubiquitin ligase (E3) is a large complex of seven or eight proteins including cdc16, 23 and 27. It is required for the separation of sister chromatids and is called the Anaphase Promoting Complex (APC).

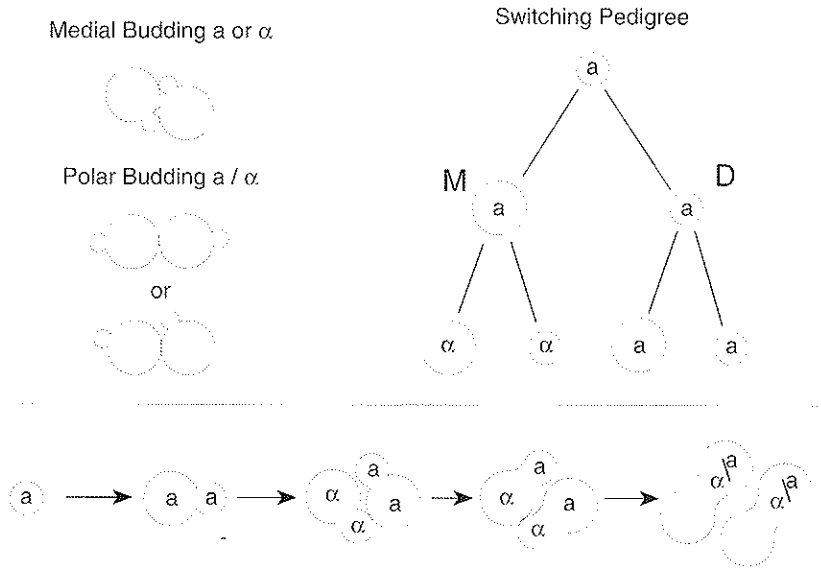

Fig. 3: Mother cell specific mating type switching facilitates diploidization of homothallic spores.

STARTING THE CELL CYCLE

Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G2.

Christian Koch

When yeast cells reach a critical size in late G1 they simultaneously start budding, initiate DNA synthesis and activate transcription of a set of genes that includes G1 cyclins *CLN1* , *CLN2*, and many DNA synthesis genes. Cell cycle regulated expression of *CLN1, 2* genes is due to the heteromeric transcription factor complex SBF (**Fig. 4**). SBF is composed of Swi4 and Swi6 and binds to the promoters of *CLN1* and *CLN2*. Different cyclin-Cdc28 complexes have different effects on late G1-specific transcription. Activation of transcription at the G1/S boundary requires Cdc28 and one of the G1 cyclins Cln1-3,

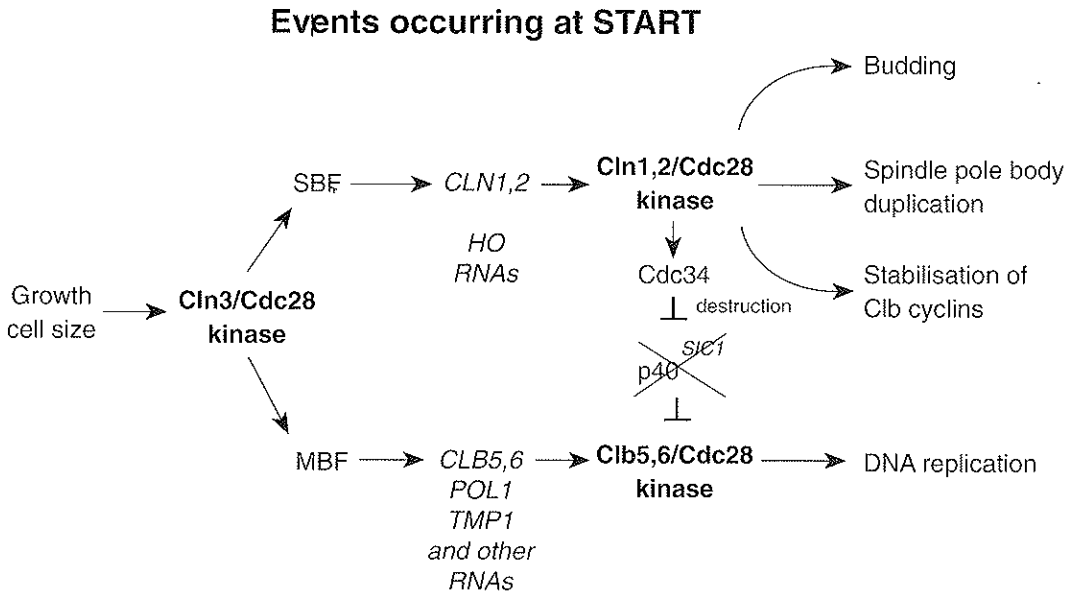

Fig. 4: Activation of cyclin dependent kinases during G1

whereas repression of SBF-regulated genes in G2 requires the association of Cdc28 with G2-specific cyclins Clbs 1-4. Using *in vivo* genomic footprinting, we show that SBF (Swi4/Swi6) binding to SCB elements (Swi4/Swi6 cell cycle box) in the *CLN2* promoter is cell cycle regulated. SBF binds to the promoter prior to the activation of transcription in late G1, suggesting that Cln/Cdc28 kinase regulates the ability of previously bound SBF to activate transcription. In contrast, SBF dissociates from the *CLN2* promoter when transcription is repressed during G2 and M phases, suggesting that Clbs 1-4 repress SBF activity by inhibiting its DNA binding activity. Switching transcription on and off by different mechanisms could be important to ensure that Clns are activated only once per cell cycle and could be a conserved feature of cell cycle regulated transcription (**Fig. 5**).

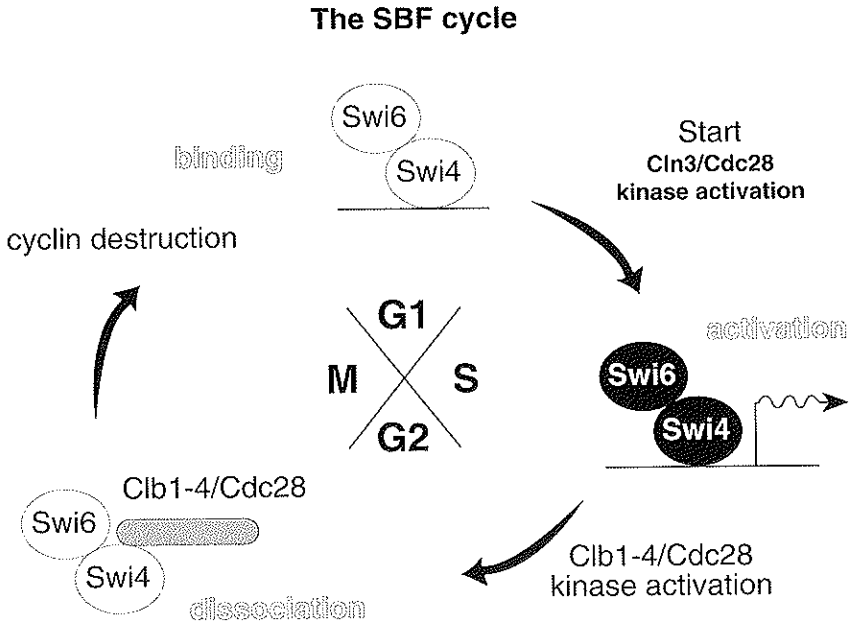

Fig. 5: Turning on and off transcription mediated by SBF

Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*

Léon Dirick, Thomas Böhm and Kim Nasmyth

In budding yeast G1 cells increase in cell mass until they reach a critical cell size, at which point (called Start) they enter S phase, bud and duplicate their spindle pole bodies. Activation of the Cdc28 protein kinase by G1-specific cyclins Cln1, Cln2 or Cln3 is necessary for all three Start events. Transcriptional activation of *CLN1* and *CLN2* by the SBF and MBF transcription factors also requires an active Cln-Cdc28 kinase and it has therefore been proposed that the sudden accumulation of *CLN1* and *CLN2* transcripts during late G1 occurs via a positive feedback loop. We report that whereas Cln1 and Cln2 are required for the punctual execution of most, if not all, other Start-related events, they are not required for the punctual activation of SBF- or MBF-driven transcription. Cln3, on the other hand, is essential. By turning off cyclin B proteolysis and turning on proteolysis of the cyclin B-Cdc28 inhibitor $p40^{SIC1}$, Cln1 and Cln2 kinases activate cyclin B-Cdc28 kinases and thereby trigger S phase. Thus the accumulation of Cln1 and Cln2 kinases which starts the yeast cell cycle is set in motion by prior activation of SBF- and MBF-mediated transcription by Cln3-Cdc28 kinase. This dissection of regulatory events during late G1 demands a rethinking of Start as a single process that causes cells to be committed to the mitotic cell cycle (**Fig. 4**).

REGULATING THE ONSET OF S PHASE IN YEAST

The B-type cyclin kinase inhibitor p40^{SIC1} controls the G1/S transition in *Saccharomyces cerevisiae*

Etienne Schwob, Thomas Böhm and Dunja Knapp

Sustained cell proliferation requires the duplication of all cell constituents followed by their segregation to two daughter cells. Most constituents are synthesized continuously throughout the inter-division period but chromosomes are duplicated during a discrete interval known as S phase. Cells ensure that chromosomes are duplicated with the same frequency as cells double their mass by control mechanisms that prevent S phase entry until cells have grown to a critical size. Experiments involving the fusion of cells at different stages of the cell cycle suggested that S phase is triggered by an inducer that is produced in late G1 as cells reach the critical size and declines later in G2.

Candidates for such an inducer were first identified by genetic studies of the budding yeast *Saccharomyces cerevisiae*. DNA replication in yeast depends on activation of the Cdc28 protein kinase through its association with G1-specific cyclins called Cln1, 2, and 3. Cdc28 kinase activity associated with Cln1 or Cln2 proteins rises in late G1 due to the transcriptional activation of the *CLN1* and *CLN2* genes. This process depends on Cln3, which seems to be present throughout G1. Premature induction of *CLN1* and *CLN2* transcription, caused either by ectopic expression or by raising Cln3 levels, triggers cells to enter S phase prematurely. This indicates that the onset of *CLN1/2* transcription is normally rate limiting for S phase entry. A similar logic may apply to the G1/S phase transition in mammalian cells, where the appearance in late G1 of cyclin E transcripts may be important.

When G1 yeast cells reach a critical size, they not only initiate DNA replication but also form buds and duplicate their spindle pole bodies, which are the first steps towards cytokinesis and chromosome segregation, respectively. Cln cyclins are needed for all three events and their function is therefore not specific to the induction of DNA synthesis. Three genes (*CDC4*, *34*, *53*) are more specifically required for S phase entry. *CDC34* encodes an ubiquitin conjugating enzyme and *CDC4* encodes a protein that contains multiple copies of a motif found in ß-transducins. Temperature sensitive mutants defective in these genes duplicate their spindle pole bodies and bud repeatedly but fail to enter S phase at the restrictive temperature. High levels of all three Cln/Cdc28 kinases accumulate in the mutants, implying that Cln kinases, though rate limiting, cannot alone trigger DNA replication.

The dependence of S phase entry on an ubiquitin conjugating enzyme (Cdc34) suggests that the G1/S phase transition needs the degradation of specific proteins. Because the sudden degradation of B-type mitotic cyclins via the ubiquitin pathway is important for exit from mitosis, it has been proposed that degradation of G1 cyclins might be required for entry into S phase and be defective in *cdc34* mutants. However, overexpression of Cln1 or Cln2 does not delay S phase, as predicted by this theory, but instead advances it. Thus, neither the activation nor the degradation of Cln cyclins seems to be the immediate trigger for S phase in yeast.

Transcripts from the B-type cyclin genes *CLB5* and *CLB6* appear in late G1 around the same time as those from *CLN1* and *CLN2*. Furthermore, S phase but not budding is delayed by at least 30 minutes in *clb5 clb6* double mutants. It is therefore possible that a rise in Cdc28 kinase activity associated with Clb5,6 might be more directly involved in activating DNA replication than increases in Cln1,2 kinases. There are two problems with this hypothesis: first, S phase is only delayed in the absence of Clb5 and Clb6 and second, premature *CLB5* expression from the *GAL1* promoter does not advance the onset of S phase.

We have shown that DNA replication in, *clb5 clb6* double mutants depends on the activity of the mitotic B-type cyclins (Clb1-4). While cells replicate with apparent normal kinetics in the absence of Clb1-4 , mutants lacking the activity of all six Clbs fail entirely to enter S phase but continue budding much like *cdc4*, *cdc34*, and *cdc53* mutants. The similar phenotypes of *cdc34* and sextuple *clb* mutants suggested that the former might be defective in activating Clb kinases. Indeed, there is little or no Cdc28 kinase activity associated with either Clb2 or Clb5 protein in *cdc34* arrested cells. A specific inhibitor of Clb/Cdc28 kinases, p40^{SIC1}, accumulates as cells exit from mitosis and normally disappears, presumably due to proteolysis, shortly before S phase. The inhibitory protein fails to disappear at the equivalent stage of the cell cycle in *cdc34* mutants and this is responsible for their failure to enter S phase. Premature expression of *CLB5* advances the initiation of S phase in mutants that lack p40. Thus, activation of Clb/Cdc28 kinases through the proteolysis of the p40^{SIC1} inhibitor may be an important step for the initiation of DNA replication in yeast (**Fig. 4**). *SIC1* mRNAs accumulate to high levels as cells exit from M phase due to transcriptional activation by Swi5.

The crucial question is what makes p40^{SIC1} a target for the ubiquitin dependent degradation ma-

chinery during the G1 to S transition. We now show that p40^{SIC1} degradation depends on CDC34 and on Clns 1 and 2. p40^{SIC1} is phosphorylated on multiple sites *in vivo* and if mutated in the three main Cdc28 consensus sequences it is hyperstable and causes cells to arrest in G1. Thus, phosphorylation of p40^{SIC1} by Serine/Threonine kinases, most likely by Cln1 and Cln2 which only appear in late G1, is crucial for the correct timing of the G1 to S transition in yeast.

HOW CELLS ENSURE THAT ORIGINS FIRE ONCE AND ONLY ONCE DURING THE CELL CYCLE

S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state

Christian Dahmann

DNA replication and mitosis are triggered by activation of kinase complexes, each made up of a cyclin and a cyclin-dependent kinase (Cdk). It had seemed possible that the association of Cdks with different classes of cyclins specifies whether S phase (replication) or M phase (mitosis) will occur. The recent finding that individual B-type cyclins (encoded by the genes *CLB1-CLB6*) can have functions in both processes in the budding yeast *Saccharomyces cerevisiae* casts doubt on this notion.

S. cerevisiae strains lacking Clb1-Clb4 undergo DNA replication once but fail to enter mitosis. We have isolated mutations in two genes, *SIM1* and *SIM2* (*SIM2* is identical to *SEC72*), which allow such cells to undergo an extra round of DNA replication without mitosis. The Clb5 kinase, which promotes S phase, remains active during the G2-phase arrest of cells of the parental strain, but its activity declines rapidly in *sim* mutants. Increased expression of the *CLB5* gene prevents re-replication. Thus, a cyclin B-kinase that promotes DNA replication in G1-phase cells can prevent re-replication in G2-phase cells. Inactivation of Clb kinases by expression of the specific Clb-Cdk1 inhibitor p40^{SIC1} is sufficient to induce a pre-replicative state at origins of replication in cells blocked in G2/M phase by nocodazole. Re-activation of Clb-Cdk1 kinases induces a second round of DNA replication.

We propose that S-phase-promoting cyclin B-Cdk complexes prevent re-replication during S, G2 and M phases by inhibiting the transition of replication origins to a pre-replicative state. This model can explain both why origins "fire" only once per S phase and why S phase is dependent on completion of the preceding M phase (**Fig. 6**).

A Dual function for Cdks: Promoting initiation of DNA replication and inhibiting pre-RC assembly

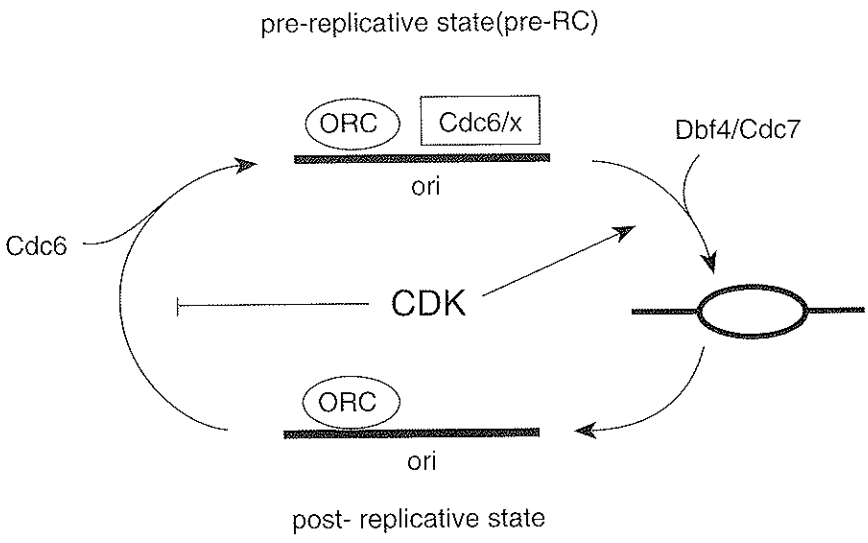

Fig. 6: The replication cycle in yeast. Initiation requires first the assembly of pre-replication complexes (pre-RCs) and second origin firing triggered by cyclin/cdk1 kinases. These kinases also inhibit *de novo* formation of pre-RCs.

Cdc6 protein is essential for the formation of pre-replication complexes at origins and can only form these complexes in the absence of cyclin B/Cdk1 kinase activity

Simonetta Piatti and Thomas Böhm

S phase entry is a highly regulated event in all eukaryotic cells. In the budding yeast *Saccharomyces cerevisiae* it is triggered by the activation in late G1 of $p34^{CDC28}$ by any one of six B-type cyclins encoded by the *CLB1-6* genes. These Clb/Cdc28 kinases are fully active in S, G2 and M, but still these cells never replicate more than once their DNA. In addition, most of the enzymes required for DNA synthesis are stable proteins present at fairly constitutive levels throughout the cell cycle. Why do S phase promoting Cdks trigger G1 nuclei but not G2 nuclei to enter S phase? Recently, it has been shown that the chromatin state of yeast replication origins differs in G1 and G2 cells. A pre-replicative state is established at the end of mitosis by the binding to origins of a putative protein complex (pre-replicative complex or pre-RC), which is then destroyed or inactivated as cells enter S phase, leaving origins in an inactive post-replicative state. This observation explains why a certain origin, once fired, cannot re-fire within a cell cycle despite the presence of S phase promoting Cdk activity. We showed, in collaboration with John Diffley's lab at ICRF, that Cdc6, an unstable protein which needs to be synthesized at each cell cycle in order to properly undergo DNA replication, is required for pre-RC formation at the end of mitosis or later in G1 (**Fig. 6**). Furthermore, Cdc6 can promote DNA replication only in a restricted window of the cell cycle: between destruction of Clbs in late anaphase and activation of Clb5/ and Clb6/Cdc28 in late G1. The latter corresponds to a "point of no return" after which Cdc6 is no longer able to promote DNA replication (**Fig. 7**). We also find that Cdc6 associates with Clb/Cdc28 kinases *in vivo* during all the stages of the cell cycle where it is inactive. These observations led us to draw a model for the regulation of S phase entry: G1 cells, which have assembled pre-RCs on origins, enter S phase upon activation of cyclinB/Cdc28 kinase, which leads to origin firing and to the switch of origins to a post-replicative state. At the same time, cyclinB/Cdc28 kinases inhibit the formation of new pre-RCs, thereby preventing re-replication. Since destruction of B-type cyclins at the end of mitosis is a prerequisite for the establishment of the pre-replicative state, this model explains the dependence of S phase on mitosis. Therefore, formation of pre-RCs and activation of SPF must occur in a defined order to ensure the correct progression through S phase. Inhibition of pre-RC assembly by the same Cdks which promote S phase entry could be important to prevent re-replication.

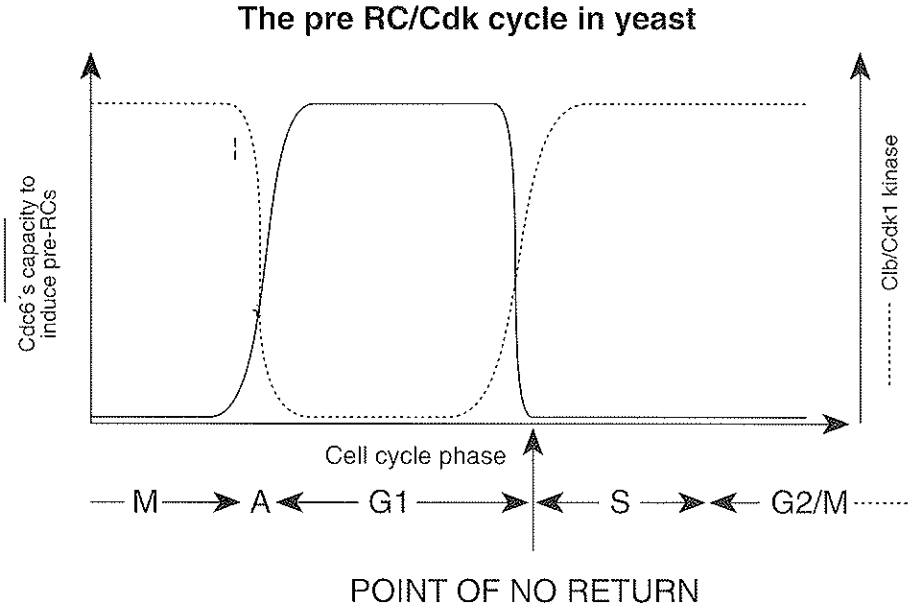

Fig. 7: Cdc6 must be synthesized during G1

MECHANISM OF CYCLIN PROTEOLYSIS

Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast

Stefan Irniger and Simonetta Piatti

Entry into M phase is caused by the appearance of active cyclin B/CDK complexes, whereas exit from M phase depends on their disappearance. During the first half of M phase, duplicated chromosomes condense and become attached via their kinetochores to mitotic spindles emanating from organizing centers (known as centrosomes or spindle pole bodies) at opposite poles of the cell. They then move to a plane that bisects the spindle, known as the metaphase plate. The latter movement, known as congression, is not an obligatory process and indeed does not occur in certain fungi. Sister chromatids remain attached to each other throughout this phase. A key transition then follows, sisters split and move to opposite poles due to two types of force exerted by the spindle: attraction of sister kinetochores towards opposite poles, known as anaphase A, and repulsion of the poles themselves, known as anaphase B. Upon completion of anaphase, mitotic spindles disassemble, chromosomes decondense, and nuclei reassemble (in animal and plant cells but not in fungi where nuclei remain intact throughout mitosis). A further round of DNA replication is not possible without the completion of mitosis. By this means, cells ensure that chromosomes cannot be re-duplicated before they have been segregated to two daughter cells.

It was thought until recently that the metaphase to anaphase transition might be triggered by destruction of B-type cyclins. This theory was based on the observation that deletion of a degenerate nine amino acid motif, RxxLxxxxN within the N termini of B-type cyclins, known as the destruction box, prevents their proteolysis and causes cells to arrest in a state resembling metaphase. However, subsequent work suggested that a lack of B-type cyclin degradation hinders not entry into but exit from anaphase. For example, expression of a version of the Clb2 B-type cyclin lacking its destruction box causes yeast cells to arrest with intact mitotic spindles and segregated chromosomes. Inactivation of mitotic CDKs due to proteolysis of their B-type cyclins is therefore thought necessary for exit from the mitotic state and also, it seems, for a further round of DNA replication.

Non-degradable B-type cyclins cause extracts from *Xenopus* eggs to arrest in a state in which the corresponding wild-type proteins are unstable and are rapidly ubiquitinated. It is therefore thought that proteolysis involves re-iterated ubiquitin conjugation and subsequent degradation by the 26S proteasome. The finding that ubiquitination of a cyclin B peptide containing a destruction box is specific to extracts from mitotic cells (in clam extracts) suggests that cell cycle control of proteolysis could be due to changes in the activity of specific ubiquitin conjugation enzymes and not due simply to changes in the state of the cyclin substrate. Changes in the activity of the proteasome during the cell cycle might also contribute. We know next to nothing about the process by which cyclin B proteolysis is initiated during anaphase, except that it requires prior activation of cyclinB/CDKs.

Mutations in a gene encoding an ubiquitin conjugating enzyme, *UBC9*, and in the genes for various proteasome subunits have been reported to cause yeast cells to arrest in G2 or M phase. However, it is not known whether this is caused by a failure to degrade B-type cyclins. All genetic approaches to mitotic cyclin degradation are beset by the problem that cyclin proteolysis is inactive for much of the cell cycle. It is therefore impossible to distinguish mutants that are genuinely defective in cyclin proteolysis from those that merely arrest at stages of the cell cycle during which proteolysis is switched off. This includes the vast majority of cell cycle mutants like, for example, mutants whose primary defect is in DNA replication.

It has recently been discovered that destruction box mediated proteolysis of the Clb2 mitotic cyclin in yeast is not confined to a short phase at the end of mitosis, but occurs also during the ensuing G1 period. Indeed, rapid Clb2 proteolysis persists until reactivation of the Cdc28 kinase due to accumulation of the G1 cyclins Cln1 and 2, which occurs shortly before S phase. Cln cyclins have an important role in budding and premature activation of Clb2 would interfere with this process and could also precipitate cells prematurely into S phase. Such a scenario is avoided by the inactivation of Clb2 proteolysis being dependent on prior activation of Clns.

The persistence of Clb2 proteolysis during G1 has the important implication that it is possible, by depriving cells of Cln cyclins, to generate synchronized yeast cultures in which all cells are active for proteolysis. In our paper, we utilize this property to isolate mutants defective in Clb2 proteolysis. We describe the identification of three genes (*CDC16*, *CDC23*, and *CSE1*) necessary for cyclin proteolysis. The proteins encoded by *CDC16* and *CDC23* are part of a multi-subunit complex composed of Cdc16p, Cdc23p and Cdc27p that is required for the onset of anaphase. B-type cyclin proteolysis is not per se needed for the metaphase to anaphase transition, but our data indicate that Cdc16 and Cdc23 may

orchestrate the proteolysis during anaphase not only of mitotic cyclins but also of proteins whose degradation is needed for sister chromatid separation (**Fig 8**). We also show that the maintenance of B-type cyclin proteolysis in G1 phase appears to have a physiological role. It is necessary to prevent DNA replication in cells arrested with mating pheromones.

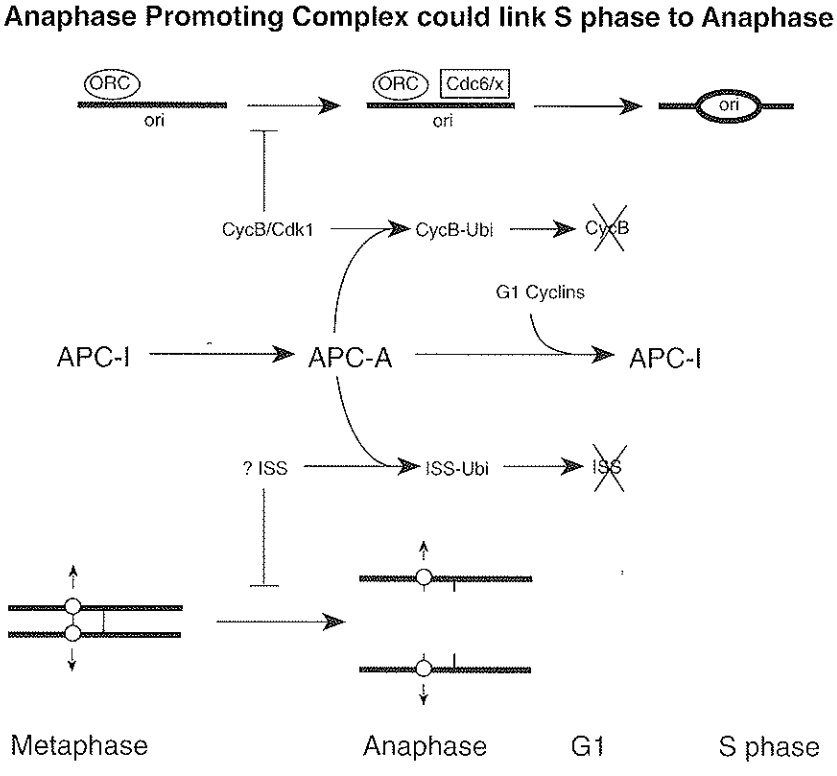

Fig. 8: The APC may link re-replication with the completion of Anaphase.

Ubiquitination of B-type cyclins depends on *CDC16, CDC23* and *CDC27*

Wolfgang Zachariae

The abundance of B-type cyclin-CDK complexes is determined by regulated synthesis and degradation of cyclin subunits. Cyclin proteolysis is required for most of the events that occur when cells exit from mitosis such as chromosome decondensation, disassembly of the mitotic spindle and cytokinesis. Regulated cyclin proteolysis is therefore a key aspect of the cell cycle clock. Recently it has been discovered that cyclin B proteolysis in yeast is activated late in mitosis and persists during the subsequent G1 period until the reactivation of the Cdc28 kinase due to the appearance of the Cln-cyclins in late G1. Three genes, *CDC16, CDC23* and *CSE1* were shown to be specifically required for cyclin B proteolysis in yeast. In extracts from frog or clam eggs, degradation is accompanied by ubiquitination of cyclin. To address whether *CDC16, CDC23* and *CSE1* are needed for cyclin ubiquitination or some other step in cyclin proteolysis we prepared extracts from G1 arrested yeast cells capable of conjugating ubiquitin to the B-type cyclin Clb2. The ubiquitination activity was cell cycle regulated, required Clb2's destruction box and was low if not absent in *cdc16, cdc23, cdc27* and *cse1* mutants. Furthermore, all these mutants were also defective in ubiquitination of another mitotic B-type cyclin, Clb3. The Cdc16, Cdc23 and Cdc27 proteins all contain several copies of the tetratricopeptide repeat and are subunits of a complex which is required for the onset of anaphase. The finding that gene products which are required for ubiquitination of Clb2 and Clb3 are also required for cyclin proteolysis *in vivo* provides the so far best evidence that cyclin B is degraded via the ubiquitin pathway in living cells. *Xenopus* homologs of Cdc16 and Cdc27 have meanwhile been shown to be associated with a 20S particle called the Anaphase Promoting Complex (APC) that appears to function as a cell cycle regulated ubiquitin-protein ligase (**Fig. 2**).

SISTER CHROMATID SEPARATION IN THE BUDDING YEAST

Christine Michaelis

During mitosis sister chromatids are separated and distributed equally to the mother and daughter nucleus. Sister chromatid separation proceeds in two steps: first the sister chromatids are separated at the metaphase/anaphase transition and then during anaphase they move towards the cell poles. Although this is one of the key steps during mitosis it has been puzzling how sister chromatids are held together during metaphase and suddenly separate during anaphase.

Destruction of B-type cyclins is important to trigger the exit from anaphase and production of non-degradable cyclin B2 arrests *S. cerevisisae* cells with separated chromatids. However, mutants which are defective in Clb2 destruction like *cdc16* or *cdc23* arrest at the metaphase stage of the cell cycle. In the absence of sister chromatids, *cdc23* mutants are able to undergo anaphase, but cannot exit mitosis (see Irniger *et al.*, 1994). Therefore it has been speculated that Cdc16 and Cdc23 are part of a general proteolysis machinery that regulates the destruction of a "glue" like protein holding sister chromatids together at metaphase and that regulates destruction of B-type cyclins at the end of anaphase.

We have isolated mutants which are defective in the cohesion of the sister chromatids in cdc16 mutants. In an initial screen we isolated chromosome loss mutants in a *cdc16-1* background by monitoring the stability of a non essential chromosome fragment containing a color marker. Cells were mutagenized with EMS and of approximately 900 000 screened colonies, 376 sectoring mutants were isolated. At the non-permissive temperature *cdc16* mutants contain one nucleus (metaphase stage). Therefore in all the *cdc16/* sectoring double mutants the nuclei and spindles were visualized and 8 mutants containing two instead of one nucleus at the non-permissive temperature were selected. We are currently cloning the genes which are defective in these mutants and will establish by FISH analysis whether the proteins are essential for sister chromatid cohesion.

The Generation of Asymmetric Gene Expression

Ralf Jansen, Nicoletta Bobola and Tae-Ho Shin

The generation of cellular heterogeneity through asymmetric cell divisions is a crucial aspect during development of multicellular organisms. However, only little is known of the molecular mechanisms involved. A well understood example of differential (asymmetric) gene activation occurs in the budding yeast *S. cerevisiae* where the *HO* gene is expressed only in mother cells but not in daughter cells. *HO* encodes an unstable endonuclease that initiates mating-type switching in budding yeast.

So far, only two genes have been implicated in the mother-specific expression of *HO*, the transcription factor *SWI5* and the repressor *SIN3*. However, *SWI5* and *SIN3* are symmetrically segregated to mother and daughter cells and not sufficient for asymmetric *HO* activation.

In a new screen we have identified 5 additional genes (*SHE1-5*) needed for mother-cell specific *HO* expression. Their function is specific to *HO* since *she* mutants express other Swi5-regulated genes normally. All *SHE* genes have been cloned. Surprisingly, *SHE1* (which is identical to the minimyosin *MYO4*) and *SHE5* code for yeast cytoskeletal proteins. The She1/Myo4, 3 and 5 proteins are localized to the bud that becomes the daughter cell. Yet, the function of the *SHE* gene products seem to be mother cell-specific. These apparently contradictory results could be explained by a model in which the She proteins (with Myo4p as a motor) transport a putative repressor of *HO* expression (or factors controlling the activity of such a repressor) from the mother to the daughter cell.

A putative *HO* repressor has been isolated by two different approaches. In both independent screens the *ASH1* gene was isolated, mutations in which lead to a very efficient *HO* expression in both mother and daughter cells. This points to a role of *ASH1* as a repressor of *HO* in daughters. Ash1 protein has been shown to be located preferentially in daughter cell nuclei. Ash1p is cell cycle regulated and present in daughter cell nuclei upon entry of the Swi5 transcription factor (necessary for *HO* expression) into nuclei of both mother and daughter cell. Ash1p might therefore block the function of Swi5 in daughter nuclei. The asymmetric distribution of Ash1p depends on functional *SHE* genes (**Fig. 9**). However, our data indicate that Ash1p is not transported by the She proteins but its expression or production is controlled by a yet unknown factor moved by the She proteins. Currently, we are trying to identify this unknown factor and to unravel the role of the individual She proteins in the transport.

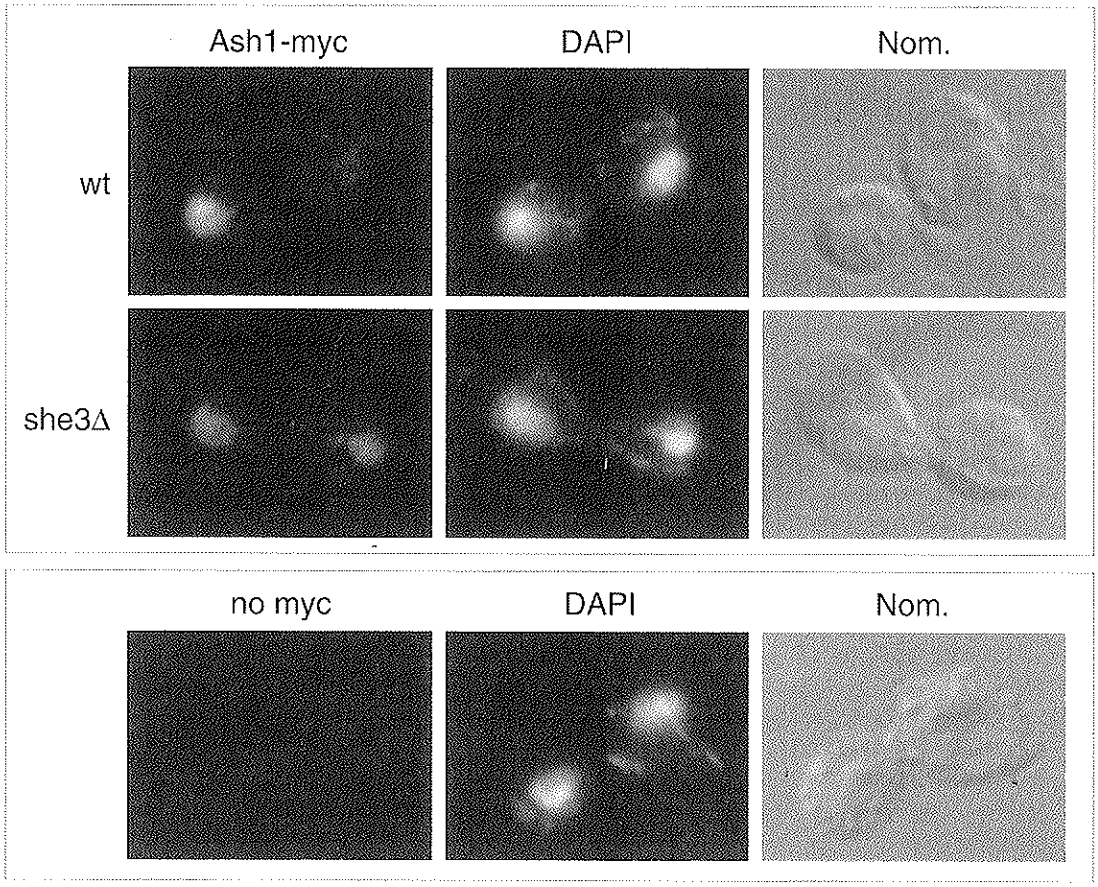

Fig. 9: Asymmetric accumulation of Ash1p depends on She proteins, one of which (She1) is a type V myosin.

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Gene function in mammalian development and oncogenesis

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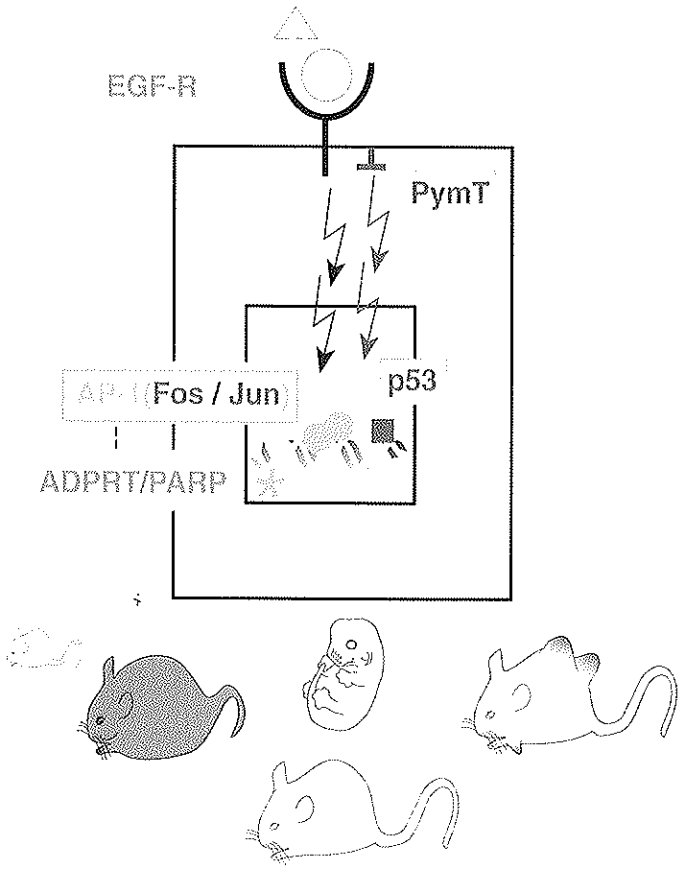

Transgenic, Chimeric & ES mice

- Tetraploid aggregation & Bone marrow reconstitution -

Fig. 1: Overview of research projects

Introduction

Growth control genes coding for growth factors and their receptors together with molecules involved in signal transduction and in gene expression are essential components for the regulation of mammalian development. We are interested in the function of such genes in normal mouse development, stem cell differentiation and tumor formation (**Fig. 1**). "Gain-of-function" (overexpression) and "loss-of-function" (gene inactivation) experiments are being used to generate genetically defined strains of mice, either by micro-injection of DNA into fertilized eggs or via mouse embryonic stem (ES) cells which are introduced into mouse embryos (ES mice). Furthermore, we are performing cell transplantations as well as gene transfer studies in various cell types including hematopoietic cells and fibroblasts. The aim of all these projects using the mouse as a model system is the better understanding of gene function in the control of normal and pathological development.

I. FUNCTIONAL ANALYSIS OF THE AP-1 TRANSCRIPTION FACTOR *IN VIVO*

Transgenic mice, embryonic stem (ES) cell chimaeras and "ES mice" generated with tetraploid blastocysts are being used to investigate the function of the AP-1 transcription factor complex. This multicomponent transcription factor appears to play a central role in controlling gene expression in cell proliferation, differentiation, development and oncogenesis. The active form of AP-1 is comprised of Jun/Fos heterodimers or Jun/Jun homodimers with its individual components (c-Fos, FosB, Fra-1, Fra-2; c-Jun, JunB and JunD). A detailed analysis of the *in vivo* function of AP-1 is being performed by generating loss-of-function and gain-of-function mutations of individual AP-1 components in mice with the aim to define the cell types and the genes which are regulated by these transcription factors.

The analysis of AP-1 mutant mice has shown that overexpression or inactivation of AP-1 proteins frequently cause deregulated cell proliferation. Experiments employing transgenes have shown that phenotypic alterations due to the loss of a specific AP-1 component can be rescued by ectopic expression of the same gene, whereas expression of a different family member generally fails to do so. These data demonstrate that the individual AP-1 components have unique and mostly non-redundant functions, whereas the precise regulation of expression of these genes is apparently not essential, even for biological processes as complex as embryonic development.

1. Cooperativity between Fos and Jun in bone tumor development

Zhao-Qi Wang and Jun Liang, in collaboration with Agamemnon Grigoriadis (London) and Karl Schellander (Vet. Univ. Vienna)

Transgenic mice overexpressing the c-Fos proto-oncogene develop osteosarcomas, whereas mice overexpressing c-Jun exhibit no phenotype. We have investigated whether Fos and Jun could cooperate *in vivo* and whether the levels of Fos are important in osteosarcoma formation. Fos-Jun double transgenic mice develop osteosarcomas at a higher frequency than single Fos transgenic mice and histological analysis indicated that these tumors contain greater quantities of neoplastic bone and a greater number of multinucleated osteoclast-like cells. Cell lines isolated from these tumors expressed besides both transgenes the matrix metalloproteinase-1 at high levels, whereas the Jun-related gene JunB was down-regulated. In contrast, overexpression of Fos in mice lacking endogenous Fos resulted in a decrease in the number of tumor-bearing mice, suggesting that a critical level of Fos is necessary for tumor development.

2. Searching for Fos target genes in osteoclasts

Koichi Matsuo

Mice lacking c-Fos exhibit a bone remodeling disease, osteopetrosis, caused by a specific block in osteoclast differentiation (Grigoriadis *et al.*, 1994). The target genes regulated by c-Fos in the osteoclast lineage are unknown. A potential target, tartrate-resistant acid phosphatase (TRAP) gene, has recently been disrupted and the resulting mutant mice also develop an osteopetrosis-like phenotype. During sequence inspection of the TRAP promoter which is highly active in osteoclasts *in vivo*, we noted that multiple binding motifs for NF-AT (**n**uclear factor of **a**ctivated **T** cells) transcription factors are con-

served in the TRAP promoter sequences from human, pig and mouse. It has been reported that NF-AT cooperates with Fos/Jun and stimulates transcription from cytokine promoters in T cells. We were able to show that human myeloid leukaemic cells HL60 express a nuclear factor which binds to the TRAP promoter motifs when *in vitro* differentiation towards osteoclasts is induced. In bandshift experiments, this binding activity is abolished by anti-Fos antibodies and by an AP-1 consensus competitor oligonucleotide. These data suggest that the binding activity to the TRAP promoter contains c-Fos. We are currently examining whether this binding activity also contains the known NF-AT components, NF-AT1, NF-AT2, NF-AT3 and NF-AT4 and is present in mouse primary osteoclasts and are also testing whether the TRAP promoter can be induced by c-Fos and NF-ATs in cultured cells.

3. The role of Fra-1 in mouse development

Martin Schreiber, Ingrid Fetka and Zhao-Qi Wang

Fra-1 is a member of the Fos gene family which, unlike other AP-1 family members, is transcriptionally controlled primarily or exclusively by AP-1 itself. To define the function of Fra-1 in mouse development, we have used gene targeting in ES cells to generate mice lacking functional Fra-1. The essential DNA binding and dimerization domains of Fra-1 were replaced by an in-frame lacZ reporter gene. Heterozygous Fra-1+/- mice are phenotypically normal and fertile, whereas embryos having both Fra-1 alleles deleted are severely growth retarded and die between E9.5 and E10.0.

Aggregation of Fra-1-/- morulae with tetraploid wild-type morulae, which can only contribute to extraembryonic tissues, prolonged the life span of the resulting chimaeric embryos up to day 17.5 of embryonic development. These data suggest that a defect in the extraembryonic compartment contributes to the observed lethality in embryos lacking Fra-1. Expression analysis using the in-frame lacZ reporter gene integrated in the Fra-1 locus is consistent with this interpretation, since Fra-1 expression in the embryo proper was detected only at day E10.5 and later. A detailed histological analysis of the placenta was performed and the labyrinthine layer was found to be reduced in size and underdeveloped. Furthermore, we observed fewer and smaller blood vessels in the yolk sacs of embryos lacking Fra-1, although fetal blood cells were apparently normal and present in both the yolk sac and the embryo itself. We are currently attempting to identify the developmental defects causing lethality of embryos lacking Fra-1.

Primary fibroblasts lacking Fra-1 were isolated from E8.5 and E9.5 embryos and subsequently established as immortalized lines following the 3T3 protocol. These cells exhibit normal proliferation and immortalization capacities compared to wild-type cells and will be further used to study the possible involvement of Fra-1 in a feedback regulation of AP-1 activity.

4. Defining the functions of c-Jun

Axel Behrens, Jun Liang, Ingrid Fetka and Zhao-Qi Wang
in collaboration with Karl Schellander (Vet. Univ. Vienna)

The proto-oncogene *c-jun* has been implicated in both cell proliferation and cell differentiation by *in vitro* and *in vivo* studies. Mice harbouring a homozygous mutation at the *c-jun* locus die between day 12.5 to 13.5 of embryonic development and it has been proposed that hepatogenesis is impaired (Hilberg *et al.*, 1993). The lethality can be rescued by a constitutive *c-jun* transgene, giving rise to viable and fertile mice with minor eye pathology and reproduction capacity. In collaboration with Kurt Zatloukal (Pathol. Inst. Univ. Graz) and Frank Hilberg (Bender & Co., Vienna) we are further characterizing the observed deficiency in liver development through analysis of chimaeras and attempting to rescue the embryonic lethality with a liver-specific albumin promoter-*c-jun* transgene. This would confirm the essential role of c-Jun in hepatogenesis and possibly uncover other sites of c-Jun's action *in vivo*. To further analyze the specificity of c-Jun we have also been able to rescue the lethality with a JunB transgene which was expressed at high levels in many cell types early in development.

To investigate the role of c-Jun in embryonic development and in the adult mouse we have decided to conditionally inactivate the *c-jun* gene using the *cre/LoxP* recombination system (Gu *et al.*, 1994 and **Fig. 2**). This strategy allows the deletion of the gene of interest in a tissue- and time-dependent manner enabling us to define c-Jun functions, e. g. in liver or in the eye in the context of the whole animal. To inactivate the *c-jun* gene in liver two mouse strains are required: One is a transgenic strain in which the *cre* transgene is expressed in a liver-specific manner (for this purpose we have chosen the albumin promoter). We have tested the albumin-*cre* construct *in vitro* and have generated transgenic lines. The

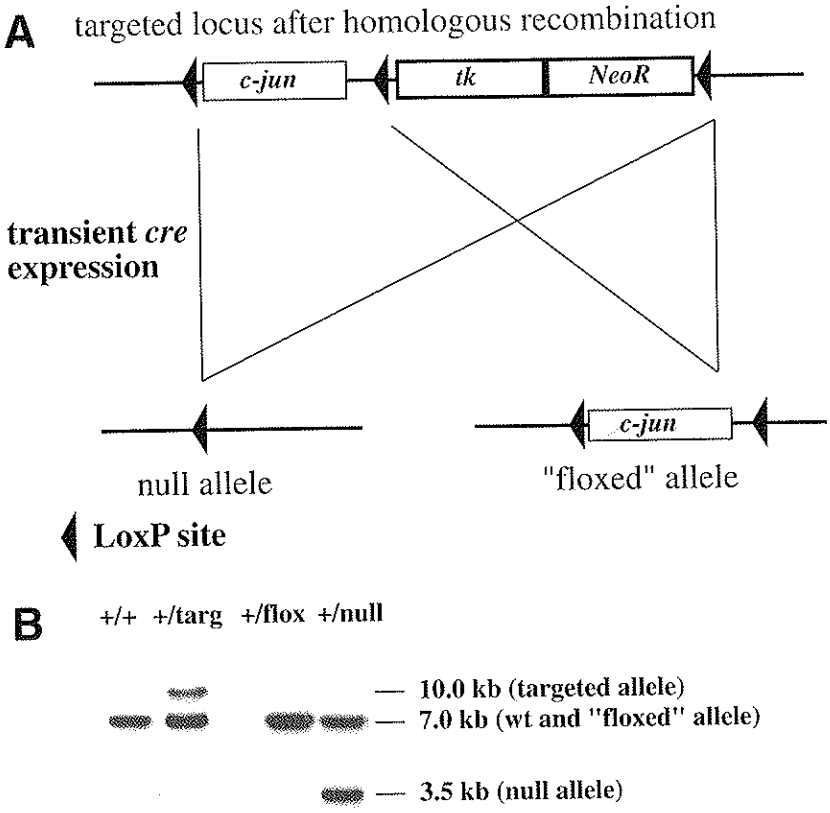

Fig. 2: Conditional inactivation of c-Jun. **A**: strategy for generation of ES cells with a c-Jun allele flanked by LoxP sites; **B**: Southern analysis of *cre*-mediated recombination at the c-Jun locus

second strain carries the *c-jun* gene flanked by two *LoxP* sites ("floxed" *c-jun*), the recognition sites of *cre* recombinase. We have obtained homologous recombination of the "floxed" targeting vector in ES cells (**Fig. 2**) and will be generating chimaeras by blastocyst injection of mutant ES cells. In offspring derived from an intercross between these strains *cre-LoxP* site-dependent recombination will occur in cells where the *cre* transgene is expressed, thereby deleting the *c-jun* gene only in liver. This approach will allow us to study the function of c-Jun not only in liver, but by crossing the "floxed" mouse with various lines expressing *cre* in a cell type-specific or developmentally stage-specific manner also in almost any other tissue of choice.

5. Functional analysis of JunB

Zhao-Qi Wang in collaboration with Marina Schorpp and Peter Angel (DKFZ Heidelberg)

The different members of the Jun protein family (c-Jun, JunB and JunD) have been suggested to exhibit specific and distinct functions in cell proliferation, differentiation and transformation. In addition to its activating function JunB is believed to negatively regulate the activity of c-Jun. Its biological role in mouse development and in particular cellular compartments is not yet defined. In order to study the function of JunB we have generated transgenic mice overexpressing the gene in many organs using promoters such as the human ubiquitinC or H2Kb. In a loss-of-function approach, we have inactivated the gene in ES cells and in mice.

Although transgenic embryos and adults expressed the transgene at high levels in many tissues, heterozygous and homozygous transgenic mice do not exhibit any obvious phenotype and are fertile. However, embryos homozygous for the disrupted *jun*B gene die at E10.0. Phenotypic alterations became visible at E7.5 as most of *jun*B-/- embryos are growth retarded. The development of the mutant embryos compared to wild-type and heterozygous

controls is delayed by one day. In addition, mutant embryos display neural defects, for example the neural tube is never closed. The histological analysis and *in situ* hybridization with specific lineage markers will further address the function of *jun*B in mouse development.

It is widely accepted that JunB and c-Jun have different functions in embryonic development and in adult tissues and that they are non-complementary. In order to analyze this phenomenon we have crossed c-*jun* and *jun*B transgenic mice to *jun*B heterozygous knock-out mice. We found that the *jun*B transgene but not c-*jun* was able to rescue the lethality of *jun*B-/- embryos. The rescued mice exhibited a developmental defect in the coat along with poor fertility. These preliminary data have to be substantiated and in the future we plan to further investigate the function of JunB in epidermal and/or hair development as well as its role in fertility.

6. Analysis of Jun and Fos functions in gene targeted fibroblasts

Martin Schreiber in collaboration with Bernd Baumann and Peter Angel, (DKFZ Heidelberg)

We have continued the analysis of AP-1 dependent processes in primary and immortalized 3T3 fibroblasts lacking c-Fos or c-Jun, focusing on cell proliferation and the mammalian UV response. In contrast to Fos-less cells, primary and immortalized fibroblasts lacking c-Jun show remarkably reduced proliferation rates and saturation densities. Attempts to link this proliferation defect to altered regulation of the cell cycle are not conclusive, since the distribution of cycling cells in different phases of the cell cycle is not significantly altered. However, the fraction of quiescent c-jun-/- cells re-entering the cell cycle within the first 20 hours after serum stimulation is reduced by about 50%. The induction of cyclin D1 mRNA by serum stimulation, which is required for G0-to-S-phase transition, is reduced in mutant cells, however, the basal levels in unstimulated cells are even higher.

Since cells lacking the tumor suppressor gene p53 behave exactly opposite to cells lacking c-Jun in many parameters related to proliferation, we have derived primary and immortalized fibroblasts lacking both these proteins. In these cells, the null mutation in p53 is epistatic to the null mutation in c-Jun: the function of c-Jun in proliferation and immortalization, which is essential in wild-type cells, is no longer required in the absence of p53 (**Fig. 3**). For example, p53-/- and p53-/-c-jun-/- cells become immortalized faster than wild-type cells, in contrast to c-jun-/- cells where the crisis is remarkably prolonged. Furthermore, cells lacking c-jun and p53 proliferate twice as fast as wild-type cells, just as p53-/- cells do, whereas cells lacking only c-Jun exhibit a reduced proliferation rate (**Fig. 3**). We will next analyze the regulation of various cell cycle molecules in c-jun-/-, p53-/- and c-jun-/-p53-/- fibroblasts to identify the molecular

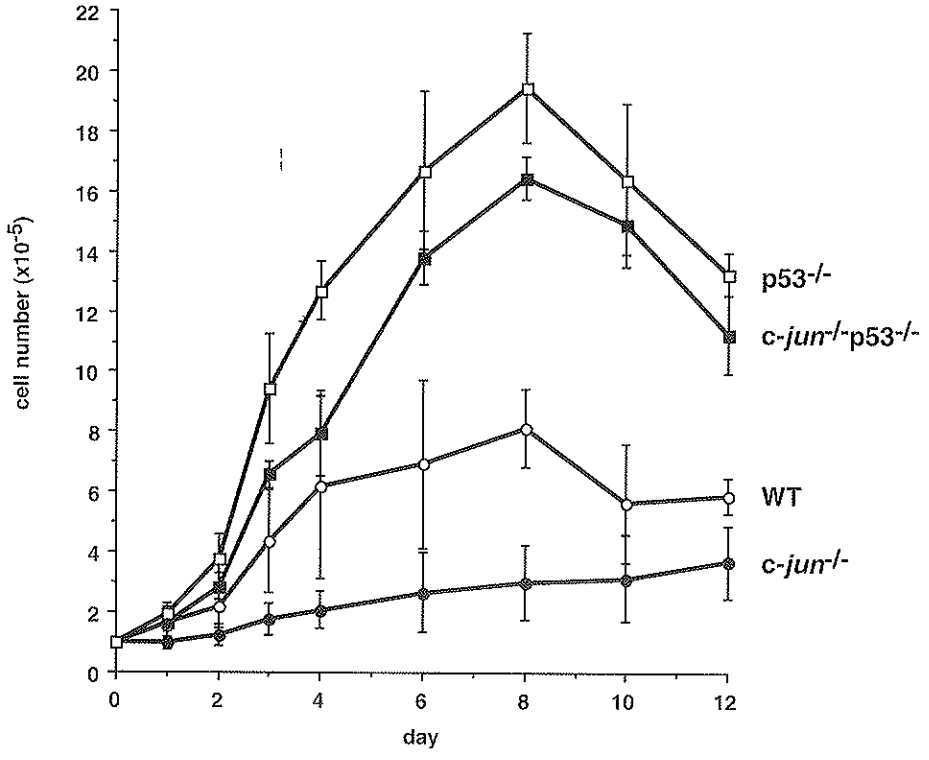

Fig. 3: p53 dependent proliferation defect of Jun-/- fibroblasts

basis of the proliferation defect in the absence of c-Jun and to elucidate if and how Jun and p53 might interact in the regulation of proliferation and the cell cycle.

UV irradiation is a potent inducer of Fos and Jun at the transcriptional and post-translational level. Therefore, 3T3 fibroblasts lacking c-Fos or c-Jun were used to demonstrate an essential function of these proteins in the response to the cytotoxic effects of short-wavelength ultraviolet (UVC) irradiation. Clonogenic survival and proliferation of these cells were drastically reduced following UV irradiation. This UV-hypersensitivity manifests itself primarily in increased cell death, largely by apoptosis, and prolonged recovery time from UV induced cell cycle arrest. Co-culture with wild-type cells did not ameliorate the hypersensitivity of c-Fos-/- cells, excluding a role for secreted factors in this effect. Transcriptional induction of the c-Fos target genes collagenase I, stromelysin-1 and stromelysin-2 by UV is almost absent in cells lacking c-Fos. These defects in target gene induction correlate with a reduced UV-induction of AP-1 DNA binding and transactivation activity. On the other hand, the repair of DNA lesions was not affected, as shown by unscheduled DNA synthesis and host cell reactivation assays. These data demonstrate that the AP-1 transcription factor (Fos/Jun) is causally involved in a novel protective function other than DNA repair against the harmful consequences of UVC.

II. CONFORMATIONAL CHANGES OF p53 AND STEM CELL DIFFERENTIATION

Sabapathy T. Kanaga

p53 is a DNA binding protein involved in regulating cell proliferation, a function which is most often abrogated by oncogenic mutations in human tumors. The p53 polypeptide has the ability to adopt two alternative conformations, defined by their differing reactivities with conformation specific monoclonal antibodies. One conformation is characteristic of wild-type p53 with a suppressing function for cell proliferation (referred to as wild-type conformation). The other (or mutant conformation) is characteristic of mutant p53 with a promoting effect on cell proliferation. Using a temperature-sensitive p53 mutant, it has been shown that cell proliferation is suppressed when p53 adopts a wild-type conformation and the suppression is relieved when the protein adopts a mutant conformation (Michalovitz *et al.*, 1990). The aim of this study is to test the hypothesis that changes in the conformation of p53 could influence the decision of cells to either undergo proliferation or differentiation. To this end, we have used embryonic stem (ES) and embryonal carcinoma (EC) cells as a model system since these cells have high proliferative capacity reminiscent of tumors, and could be induced to differentiate upon suitable stimuli *in vitro* and *in vivo*.

Initial screening by immunoprecipitation of pulse labeled undifferentiated ES and EC cell lysates with conformation specific antibodies revealed that these cell lines predominantly have the p53 protein in a wild-type conformation. Treatment of various ES and EC cell lines with differentiating agents like retinoic acid and DMSO resulted in a shift towards a mutant conformation. We next determined if this conformational shift upon differentiation could be correlated with p53 functional activity, i.e. (i) specific DNA binding, (ii) transactivation of p53 responsive promoters and (iii) mitotic spindle checkpoint arrest. Preliminary data indicate that p53 binds DNA specifically only in undifferentiated ES cells, where p53 was capable of transactivating stably integrated p53 responsive promoters; this transactivation potential was lost upon differentiation. Furthermore, undifferentiated ES cells could execute mitotic spindle checkpoint when treated with colcemid, whilst this potential is lost in differentiated cells reminiscent of p53 null fibroblasts.

To ascertain the physiological significance of p53 in the wild-type conformation, we have subjected the ES cells to genotoxic agents like UV and γ-irradiation. UV irradiated undifferentiated CCE cells are killed within the first six hours as determined by LDH release assays. In contrast, the differentiated CCEs and both undifferentiated and differentiated p53 null ES cells are resistant to UV up to 12 hours. This suggests that the function of p53 in undifferentiated cells is to protect the integrity of the genome and prevent propagation of mutations, which may be possible in differentiated cells.

These data indicate that p53 in the wild-type conformation in undifferentiated ES cells is functionally active and this activity is lost upon differentiation. At present, we are attempting to express the p53 protein in either of these conformations in p53 -/- ES cells to investigate if the enforced expression of a specific conformation would influence the decision of a cell to proliferate or differentiate.

III. ROLE OF ADPRT (PARP) IN GENOMIC STABILITY AND IN APOPTOSIS

Zhao-Qi Wang and Laura Stingl in collaboration with Michael Jantsch
(Department of Cytology and Genetics, Vienna University)

ADPRT (PARP), which catalyzes poly-ADP-ribosylation of nuclear proteins, is thought to play an important role in chromosomal stability, DNA repair, cell proliferation and apoptosis. To elucidate the function of ADPRT, the gene was inactivated in mice. While young ADPRT-/- mice display no phenotypic abnormalities, older mice originating from a mixed genetic background (129/Sv x C57BL/6) are susceptible to epidermal hyperplasia. However, mutant mice are resistant to DMBA/TPA-induced skin papilloma formation. Another age-related phenotype was obesity which developed in 50% of mutant female mice (129/Sv x C57BL/6) older than 14 months. Obese mice had slightly increased serum glucose levels, suggesting a role for ADPRT/ADP-ribosylation in general metabolism.

We have analyzed the consequences of the absence of ADPRT at the cellular level in regard to DNA repair, proliferation, apoptosis as well as chromosomal stability. While ADPRT-/- fibroblasts display normal DNA repair capacity, the cells grow more slowly *in vitro*. This difference was more pronounced at 39°C of the culture, implying that the lack of ADPRT may either affect cell proliferation or sensitize the cells to experimental stress. An effect on cell proliferation was also observed *in vivo* in chimaeric fetuses generated by aggregating wild-type and mutant embryos. Using ADPRT-/- pancreatic islet cells we were able to show, in collaboration with H. Kolb's group (Düsseldorf) that NAD+ depletion was a cause for free radical-induced cell death which was believed to be dependent on the activation of ADPRT.

To study the role of ADPRT in apoptosis, mutant cells were treated with different reagents, like Fas antibody and TNFα, which induce apoptosis via different pathways. The results showed no difference between wild type and mutant cells in apoptosis, indicating that the role of ADPRT during apoptosis is not a signal triggering the apoptotic program. Since ADPRT is a highly abundant, chromatin-associated protein and many nuclear proteins are acceptors for poly-ADP-ribosylation, it is thought to play a role in chromosomal stability and/or recombination. To investigate this, we performed a micronuclei assay, which measures the stability of chromosomes and analyzed the frequency of sister chromatid exchanges (SCE). Compared to wild-type cells, ADPRT-/- splenocytes contained more micronuclei and significantly higher SCEs (2-3 fold, see **Fig. 4**), indicating an important role for ADPRT in genomic stability.

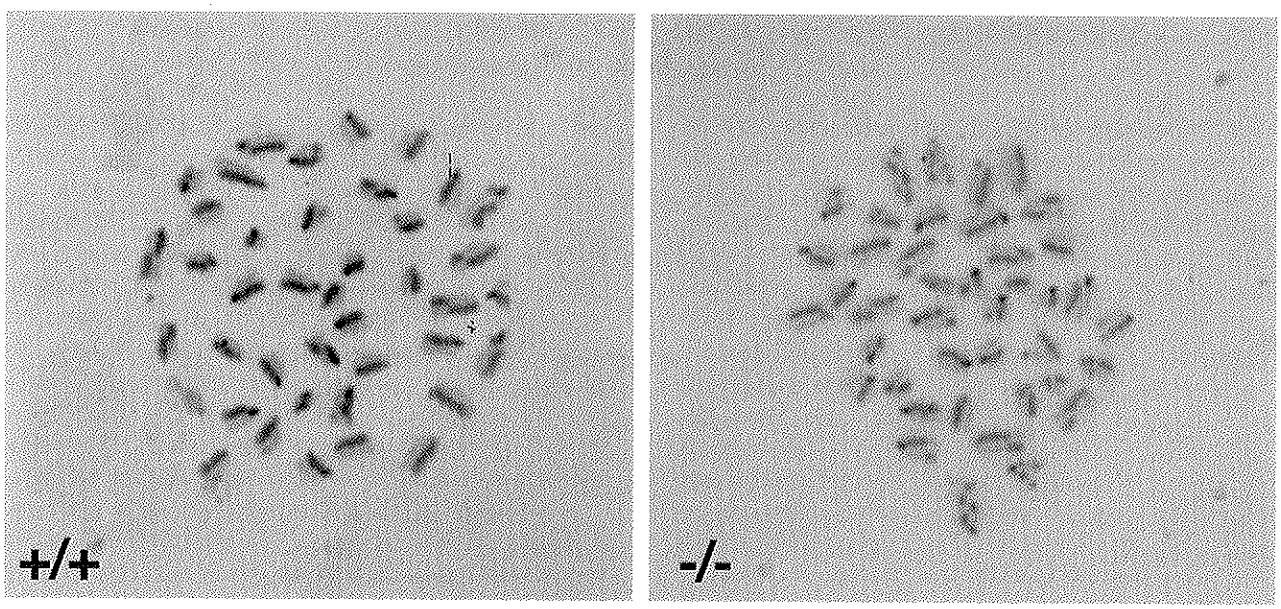

Fig. 4: Frequency of SCEs in wild-type (+/+) and ADPRT-/- splenocytes

IV. POLYOMA MIDDLE T IN ENDOTHELIAL TUMOR DEVELOPMENT

Sabapathy T.Kanaga, Uta Möhle-Steinlein, Ulrich Mühlner and Ingrid Fetka

The middleT antigen of murine polyomavirus (PymT) binds to and activates several protein tyrosine kinases of the Src family. *In vivo*, PymT rapidly transforms endothelial cells leading to vascular malformations reminiscent of endothelial tumors or hemangiomas. We have shown that PymT-transformed endothelial cells (END cells) derived from such tumors efficiently induce vascular lesions by host cell recruitment and lead to the formation of secondary tumors. In addition, END cells exhibit altered proteolytic activity and show increased expression of urokinase plasminogen activator (uPA) while plasminogen activator inhibitor (PAI-1) expression is drastically reduced.

Since Src family tyrosine kinases are thought to be involved in mediating the oncogenic activity of PymT, we have shown that inactivation of a single tyrosine kinase does not influence the induction of vascular malformations. However, it appears that the PymT-Yes kinase complex may play a major role in the initiating action of PymT (Kiefer *et al.*, 1994). We are at present attempting to produce double kinase mutant mice to investigate if PymT could transform endothelial cells in the absence of two known kinases. Furthermore, we have shown by Northern analysis that the endothelial cell specific tyrosine kinase receptor, Flk-1, is efficiently expressed in END cells. Its ligand, VEGF, was also found to be expressed by END cells using Northern analysis and RT-PCR. Furthermore, VEGF-165 was detected by Western blot analysis from conditioned medium of END cells, suggesting that a VEGF-Flk-1 autocrine loop could be responsible for the proliferation of these cells. We are currently testing this possibility, and are attempting to produce conditional Flk-1 knock-out mice (in collaboration with W. Risau, Bad Nauheim) to ascertain its role in PymT-induced endothelial cell transformation.

To define a causal role of plasminogen activators (PA), which convert plasminogen to active plasmin, in matrix degradation during the formation of vascular lesions, we have used knock-out mice lacking uPA, tPA, PAI-1, utPA and plasminogen, and found that none of these components are absolutely required for PymT induced endothelial cell transformation. However, PA mutant END cells have altered growth rates in fibrin gels (in collaboration with M. Pepper, Geneva) and exhibit a reduced frequency of tumor formation *in vivo*. In reciprocal experiments, the PA mutant mice were found to have altered tumor formation rates and incomplete penetrance when inoculated with wild-type END cells. These data suggest that there may be another pathway leading to the degradation of matrix in the PymT induced endothelial cell transformation process and that a balanced proteolytic activity of both the host and the introduced END cells is necessary but not sufficient for the formation of vascular tumors.

V. EGF-R FUNCTION IN PROLIFERATION AND DIFFERENTIATION

Maria Sibilia and Alexander Fleischmann

The epidermal growth factor receptor (EGFR) belongs to a family of tyrosine kinase receptors and is activated by several ligands including EGF and TGFα. In the mouse blastocyst, EGFR is detected on the trophectoderm and later in gestation EGF binding activity can be detected in various organs. Amplification and overexpression of the receptors have been observed in human carcinomas and brain tumors.

In order to examine the importance of EGFR in proliferation and differentiation, mice and cells lacking functional EGFR were generated by inactivating the gene in ES cells. Homozygous mutant mice are retarded in growth and die at different stages of embryonic development depending on their genetic background. In an inbred 129/Sv background, mutant mice die at midgestation, whereas in a mixed 129/Sv x C57BL/6 they can survive until birth and even to postnatal day 20 in a 129/Sv x C57BL/6 x MF1 background (**Fig. 5**). Death in utero probably results from a defect in the placentas, which are smaller in size and have an underdeveloped spongiotrophoblast layer. We have rescued the placental defect by generating aggregation chimaeras between EGFR mutant and tetraploid wild-type embryos, the latter contributing exclusively to the extraembryonic tissues. Under these conditions, viable EGFR mutant mice were obtained in a pure 129/Sv genetic background which showed similar defects as the mutants in the other backgrounds (see below). These results indicate that the genetic background most likely acts on the development of the extraembryonic tissues and we are currently analyzing the placental defect more precisely in order to understand the mechanisms responsible for the strain-dependent lethality.

At birth, all mutant mice have open eyes, rudimentary whiskers and defects in various epithelia like lungs and epidermis. The lung immaturity is most likely responsible that the majority of mutant newborns cannot initiate or sustain respiration. Using an *in vitro* organ culture system (in collaboration with Heber Nielsen, Boston) we are currently measuring the development of lung branching morphogenesis in EGFR mutant embryos.

After postnatal day 10, all mutant mice develop

several brain defects, the most severe being atrophy of the entire cerebral cortex which resembles ischemic infarction. In collaboration with Adriano Aguzzi, Zürich and Derek van der Kooy in Toronto we are trying to identify which cell types in the brain are affected and responsible for the observed phenotypes.

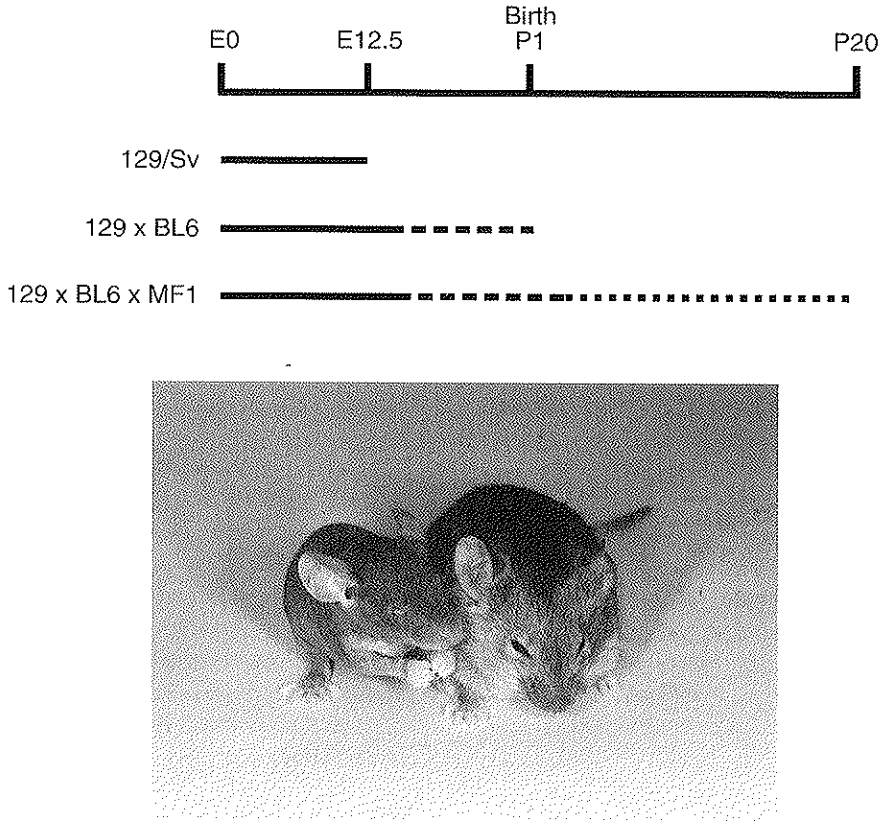

Fig. 5: EGRF mutant mice die at different stages of development depending on their genetic background. Top scheme illustrates the viability of mutant mice in 3 different mouse strains during embryonic and postnatal development. The lower picture shows an EGFR mutant mouse (left) with its control littermate (right) at postnatal day 18 where the severe growth retardation, the eye and skin lesions are visible.

Analysis of ES cells and fibroblasts lacking EGFR

In vitro differentiation of ES cells provides a powerful model system for studying the function of genes in cell differentiation. Under appropriate conditions, ES cells can be induced to differentiate into cells of multiple cell types including cells of the hematopoetic, endothelial, muscle and neuronal lineages. We are interested in whether the loss of EGFR affects the differentiation capacity of ES cells, in particular with respect to neuronal differentiation. An essential role for EGFR in neuronal differentiation was suggested by the *in vivo* data as well as by several *in vitro* studies using dominant negative receptors. Preliminary data suggest that ES cells lacking both EGFR alleles are significantly impaired in their ability to differentiate into neuron-like cells. Further studies using antibodies for specific marker will aim to show whether the effect is restricted to specific cell types within the ensemble of cells demonstrating neuron-like morphology.

Primary embryonic fibroblasts lacking EGFR were isolated, and spontaneously immortalized cell lines were established. We will next investigate how downstream signaling is affected in these cells and also analyze several cellular processes in which EGFR seems to be involved, such as proliferation, transformation and the UV response.

VI. GENERATION OF ES MICE

Zhao-Qi Wang

We have continued to develop an efficient technique to generate ES mice directly from manipulated ES cells using tetraploid embryo donors (see Report 1994). We successfully applied this technique to various wild-type ES cells (R1 from A. Nagy and GS1 kindly provided by M. Aguet) as well as to selected R1 (Fra-1+/-) cell clones. We believe that this method is very useful for shortening the time necessary to generate mice with predetermined genetic changes and we are planning to expand these studies to other ES cell lines.

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Regulation of protein serine/threonine kinases

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Introduction

Addition of mitogens to quiescent cells induces a number of responses that together result in DNA synthesis and cell division. One of the earliest mitogenic responses is the phosphorylation of ribosomal protein S6. S6 phosphorylation is thought to facilitate an increased rate of synthesis of certain proteins, such as ribosomal proteins and protein synthesis elongation factors, that are required for efficient G_1 progression and whose mRNAs contain a polypyrimidine tract at the 5' end (Jefferies *et al.*, 1994). Two families of mitogen-stimulated S6 kinases have been identified: the M_r=70/85,000 S6 kinases ($p70^{S6k}$/$p85^{S6k}$) and M_r=90,000 ribosomal S6 kinase ($p90^{rsk}$). Enzymes in both families are activated by phosphorylation of Ser/Thr residues. $p90^{rsk}$ is activated by mitogen-activated protein (MAP) kinases (Sturgill *et al.*, 1988) and participates in a network that can include Ras, Raf-1 and Mek1 (**Fig. 1**). By contrast, $p70^{S6k}$ lies on a distinct pathway that does not include MAP kinases (Ballou *et al.*, 1991; **Fig. 1**). In the last year, significant progress has been made in identifying some of the signaling components that function in the second pathway.

$p70^{S6k}$ is the major S6 kinase activity in mouse fibroblasts. The protein exists as two isoforms (M_r=70,000 and 85,000) which are identical except that $p85^{S6k}$ has an additional 23 amino acids at the N terminus that act as a nuclear targeting sequence. *In vivo* $^{32}P_i$ labeling and sequencing of phosphopeptides led to the identification of four mitogen-induced phosphorylation sites clustered at the C terminus of $p70^{S6k}$ (Ferrari *et al.*, 1992; **Fig. 2**). These sites occur within the motif Ser/Thr-Pro, which is a consensus sequence for phosphorylation by MAP kinases and cyclin-dependent kinases. However, there is no evidence yet that enzymes in these families activate $p70^{S6k}$. In addition to the four C-terminal phosphorylation sites, $p70^{S6k}$ also contains additional phosphates that are essential for enzyme activity (**Fig. 2**). Rapamycin, an immunosuppressant that arrests the growth of some cell types in G_1, induces the dephosphorylation of some of these sites and therefore prevents the activation of $p70^{S6k}$ (Han *et al.*, 1995). The presence of these different phosphate groups suggests that two or more phosphorylation events might be involved in regulating the activity of the enzyme.

The main focus of our research has been to (a) identify the kinases that activate $p70^{S6k}$; (b) identify additional upstream regulators in the $p70^{S6k}$ signaling cascade; (c) determine what role $p70^{S6k}$ plays in cell growth and the cell cycle; and (d) identify additional physiologically important substrates of $p70^{S6k}$.

Identification of S6 kinase kinases

Christian Kühne and Pavel Kovarik

In analogy with the mitogen-induced pathway leading to the activation of $p90^{rsk}$, it appears that $p70^{S6k}$ participates in a phosphorylation cascade with at least one Ser/Thr-specific kinase acting upstream of $p70^{S6k}$ (**Fig. 1**). We have used a variety of biochemical and genetic strategies to identify such an S6 kinase kinase. In the past, phosphatase-treated $p70^{S6k}$ purified from fibroblasts was used as a substrate in an *in vitro* reactivation assay to screen fractionated cell extracts and purified enzymes. This approach was not successful because the fully dephosphorylated enzyme loses its secondary structure. The best substrates to search for mitogen-activated or constitutively active S6 kinase kinases would probably be wild-type and mutant peptides containing the known phosphorylation sites in $p70^{S6k}$ (see **Fig. 2**).

In a genetic approach to identify kinases that modify $p70^{S6k}$, we used yeast two hybrid screening to find proteins that bind to the enzyme *in vivo*. We

screened 4x10^6 independent yeast transformants and isolated 111 positive clones that fell into four groups. Class I clones encode the 3' end of an unknown gene. Class II clones also encode the 3' end of an unknown gene but it has some sequence similarity to a known kinase subunit. Antiserum to the putative protein fragment has been made and tested on Westerns and in immunoprecipitation kinase assays. Attempts to obtain a full-length class II clone have so far not been successful. Class III clones encode a DNA-binding protein of unknown function. This protein could be involved in the nuclear localization of p70^{S6k} or p85^{S6k} (see below). Finally, class IV clones code for a known kinase subunit. This kinase phosphorylates p70^{S6k} at sites distinct from the mitogen-activated sites. One of these phosphorylation sites was mapped and stable cell lines expressing p70^{S6k} constructs mutated at this site were made. A preliminary analysis of the regulation of the activity of the mutant kinase has been done. We will continue to concentrate on characterizing the class II and IV gene products and determine whether they are involved in regulating p70^{S6k} function.

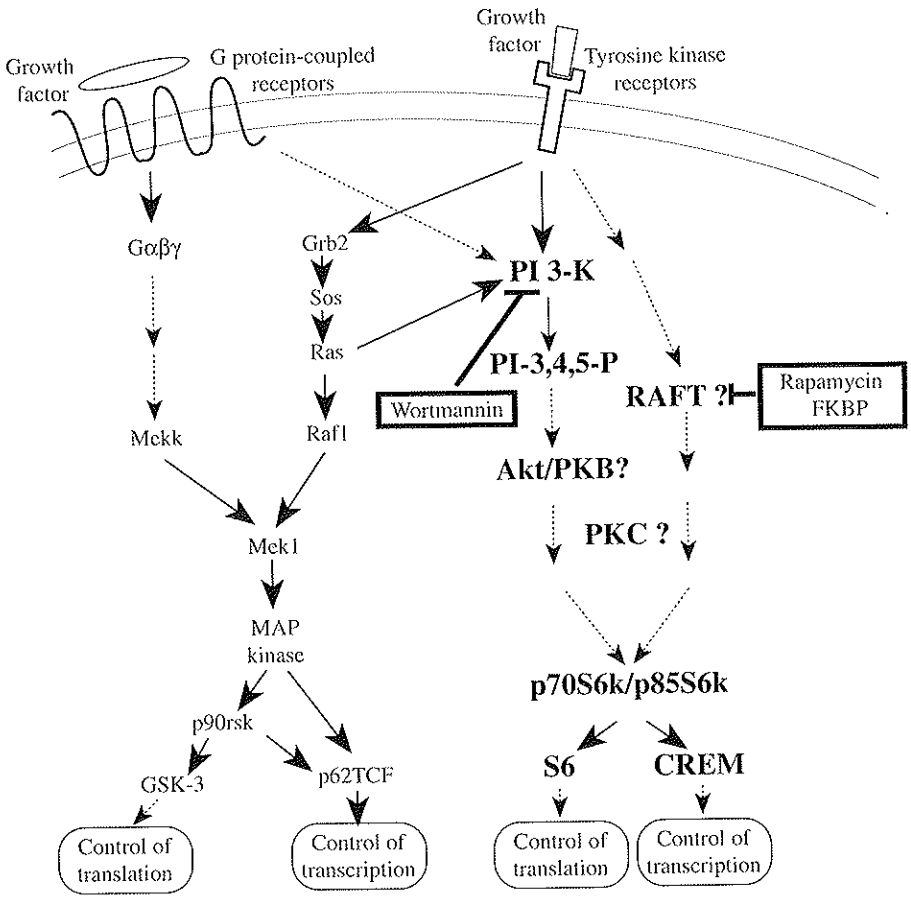

Fig. 1: Signal transduction pathways leading to the activation of p90^{rsk} and p70^{S6k}.

Upstream regulators of p70^{S6k}

Claudia Petritsch (in collaboration with Peter Parker and Rüdiger Woscholski, ICRF, London)

Another approach to identify components in the p70^{S6k} pathway is to study the mechanism of action of inhibitors of the pathway. We found that pretreatment of fibroblasts with certain cyclic nucleotide phosphodiesterase inhibitors (theophylline, SQ 20,006 and MY-5445) reversibly blocks the phosphorylation and activation of p70^{S6k} but has no effect on p90^{rsk} or MAP kinases (Petritsch *et al.*, 1995a). Our initial hypothesis to explain the effect of these compounds was that they increase intracellular cyclic nucleotide levels, and as a result cyclic nucleotide-dependent kinases are activated and phosphorylate a regulatory

protein in the $p70^{S6k}$ pathway. It has been reported that cAMP antagonizes $p70^{S6k}$ activation in T cells by such a mechanism (Monfar *et al.*, 1995). However, a distinct collection of cAMP and cGMP agonists and analogues did not suppress $p70^{S6k}$ activation in fibroblasts, indicating that (a) high intracellular cyclic nucleotide concentrations do not antagonize the $p70^{S6k}$ pathway and (b) phosphodiesterase inhibitors block $p70^{S6k}$ activation by a mechanism that is independent of cAMP or cGMP production (Petritsch *et al.*, 1995a). The effect of theophylline and SQ 20,0006, but not MY-5445, on $p70^{S6k}$ signaling may be due in part to the inhibition of a phosphatidylinositol (PI) 3-kinase that acts upstream of $p70^{S6k}$ (see below). Finally, in contrast to many other cell types, cAMP and cGMP were also found to have no inhibitory effect on MAP kinase/$p90^{rsk}$ activation in mouse fibroblasts (Petritsch *et al.*, 1995a). Thus, cyclic nucleotide-dependent signaling pathways may interact in a variety of ways with the $p70^{S6k}$ and MAP kinase/$p90^{rsk}$ pathways. These interactions may be important for generating cell type-specific responses to identical physiological conditions.

Rapamycin, when bound to its intracellular receptor FKBP, also blocks the activation of $p70^{S6k}$. It was shown earlier that rapamycin suppresses the growth of yeast by interacting with two gene products encoded by *TOR1* and *TOR2* (Kunz *et al.*, 1993). Homologous proteins (RAFT) were subsequently found in higher eukaryotes. These proteins exhibit significant homology to the catalytic subunit of mammalian PI 3-kinase, which plays an important role in mitogenesis and other cellular responses. Although it was not demonstrated biochemically that the TOR1 and TOR2 proteins phosphorylate inositol lipids, these results suggested that PI 3-kinase or a related enzyme might act upstream of $p70^{S6k}$ (**Fig. 1**). We tested this hypothesis by using two potent and irreversible inhibitors of PI 3-kinase, wortmannin and demethoxyviridin, to probe the $p70^{S6k}$ activation pathway. We found that treatment of fibroblasts with either of these structurally related compounds irreversibly blocked the mitogen-induced activation of $p70^{S6k}$ (Petritsch *et al.*, 1995b). By contrast, activation of $p90^{rsk}$ and MAP kinases was not affected. IC_{50} values of 45 nM (wortmannin) and 400 nM (demethoxyviridin) were obtained when EGF was used as an S6 kinase activator; with PDGF, the IC_{50} values were about three times higher. These results might indicate that the PDGF and EGF receptors signal through different forms of PI 3-kinase, with the former being more resistant to wortmannin and demethoxyviridin. In addition to EGF and PDGF, $p70^{S6k}$ can be activated by a wide variety of agents that act through different mechanisms. We found that pretreatment of fibroblasts with wortmannin reduced $p70^{S6k}$ activation by each agonist tested, but to different extents. The inhibition was lowest with phorbol 12-myristate 13-acetate and bombesin, and highest with the Ca^{2+} ionophore A23187, insulin and EGF. The relative resistance to wortmannin seen with some $p70^{S6k}$ agonists suggests that PI 3-kinase-independent signaling pathways might also be involved in S6 kinase activation. Finally, some S6 kinase agonists not previously known to activate PI 3-kinase (A23187, bombesin and phorbol 12-myristate 13-acetate) were found to increase the production of PI 3,4,5-trisphosphate in a wortmannin-sensitive manner (Petritsch *et al.*, 1995b).

The results above support a model in which PI 3-kinase acts upstream of $p70^{S6k}$ in a mitogenic signaling cascade. A similar conclusion has been reached by others through the use of distinct PI 3-kinase inhibitors, PDGF receptor mutants defective in PI 3-kinase binding, and a constitutively active form of PI 3-kinase. Furthermore, it appears that a kinase encoded by the *Akt* protooncogene may be a downstream target of PI 3-kinase (Burgering *et al.*, 1995; **Fig. 1**). It has not yet been tested whether the akt kinase might be inhibited by MY-5445 or other compounds that inhibit $p70^{S6k}$ activation. Finally, it appears that the rapamycin-sensitive signal transmitted to $p70^{S6k}$ via RAFT represents an independent pathway that contributes to S6 kinase activation (**Fig. 1**).

Cell cycle regulation of $p70^{S6k}$ and MAP kinases

Helga Edelmann

$p70^{S6k}$ is activated as much as 50-fold upon addition of mitogens to cells, and two lines of evidence suggest that this activity is important for cell cycle progression. First, microinjection of antibodies that inhibit $p70^{S6k}$ into fibroblasts at any time during G_1 blocks entry into S phase (Lane *et al.*, 1993). Second, treatment of cells with rapamycin leads to either cell cycle arrest in G_1 or a delay of entry into S phase, depending on the cell type. Activation of MAP kinases is also thought to be essential for triggering the proliferative response in fibroblasts. To gain more insight into how these kinases are regulated and what roles they might play in controlling cell growth and the cell cycle, we examined the behavior of $p70^{S6k}$ and MAP kinases during the cell cycle. Using highly synchronized populations of fibroblasts, we found that the kinases are activated not only at the G_0/G_1 boundary, but also in cells progressing from M into G_1 after release from a metaphase block (Edelmann *et al.*, 1996). $p70^{S6k}$ was activated 20-fold when cells entered G_1 from a quiescent state and the activity remained high throughout G_1. During S and

G_2 the activity decreased constantly, so that during M phase a low level of S6 kinase activity remained. The kinase was reactivated 10-fold when mitotic cells entered G_1 of the next cell cycle. In contrast, MAP kinases were active only very early during the G_0/G_1 transition and then were reactivated at the end of M phase. This regulation appeared to be mediated mainly through post-translational mechanisms, as protein levels did not change significantly (Edelmann *et al.*, 1996).

In addition to changes in activity, immunofluorescence studies showed that $p70^{S6k}$ changes its intracellular localization during the cell cycle (Edelmann *et al.*, 1996). Bright speckles of $p70^{S6k}$ staining appeared in the nucleus of S phase cells, and during mitosis the enzyme colocalized with chromosomes. Since staining of the cytoplasm remained about the same during all phases of the cell cycle, the increased signal in the nucleus could be due to concentration of nuclear $p70^{S6k}$ into localized spots rather than to an influx of cytoplasmic enzyme. The antibody used in these experiments detects both $p70^{S6k}$ and $p85^{S6k}$, so no statement can be made about which isoform is present in the speckles.

It was proposed that phosphorylation of four amino acids at the C terminus of $p70^{S6k}$ might be responsible for mitogen-induced enzyme activation at G_0/G_1 (Ferarri *et al.*, 1992; see **Fig. 2**). To test this hypothesis, a mutant $p70^{S6k}$ in which the three Ser residues were mutated to Asp and the Thr to Glu was stably expressed in fibroblasts. These changes were introduced to mimic phosphorylation. If the four C-terminal phosphorylation sites are responsible for regulating the activity of $p70^{S6k}$ during the cell cycle, the mutant kinase should display the same level of activity at all times. However, we found that the mutant kinase was still activated normally during G_0/G_1 and M/G_1 (Edelmann *et al.*, 1996). Furthermore, the enzyme also remained sensitive to negative regulators of the $p70^{S6k}$ pathway such as wortmannin, rapamycin and serum withdrawal. Thus, the apparently normal regulation of the $p70^{S6k}$ mutant suggests that the C-terminal phosphorylation sites alone do not regulate $p70^{S6k}$ activity during the cell cycle. The same conclusion was reached by others, using a $p85^{S6k}$ mutant with 104 amino acids deleted from the C terminus. Furthermore, it was proposed that kinase activation might result from phosphorylation of Thr229 in response to a signal generated by PI 3-kinase (Weng *et al.*, 1995). The specific mechanisms that contribute to the cell cycle-dependent regulation of $p70^{S6k}$ activity remain to be determined.

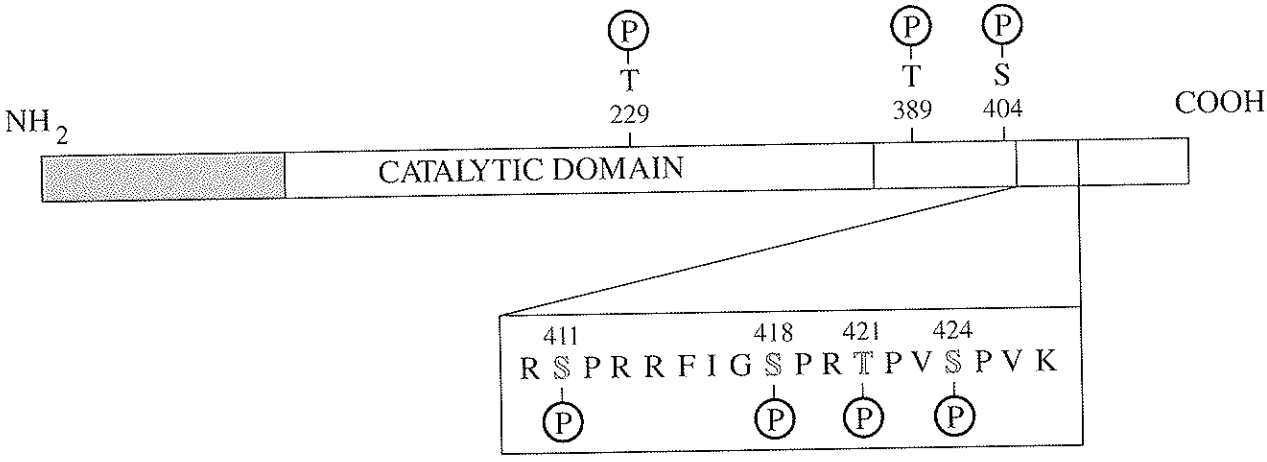

Fig. 2: Structure of $p70^{S6k}$ showing location of known phosphorylation sites. Mitogen-induced sites at the C terminus are boxed (Ferarri *et al.*, 1992). Thr229 is phosphorylated in response to PI 3-kinase (Weng *et al.*, 1995). Phosphorylation of Thr229, Thr389, Ser404 and Ser411 is rapamycin-sensitive (Han *et al.*, 1995).

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Mammalian developmental genetics

Mouse mutants as tools to isolate developmental control genes

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Introduction

Intra-uterine growth of the mammalian embryo depends on implantation into the maternal uterus and the formation of a placenta that permits maternal-fetal interactions throughout the period of gestation. Growth control mechanisms are necessary, not only to transfer sufficient maternal resources to the fetus to allow complete development, but to limit this transfer to an extent that is compatible with maternal survival and the sucessful birth of a mature fetus. A large number of genes with positive and negative actions on growth would be involved in this process, some would act on the placenta, and others directly on the embryo. In addition to normal growth control genes, there is considerable evidence that genes subject to gametic imprinting (i.e., genes whose expression is influenced by parental transmission) also play a role in the control of embryonic growth. The primary goal of our research is to clone and characterize genes involved in processes that regulate growth of the mammalian embryo in utero. We are attempting this in two ways, both of which rely on mouse mutants to provide a molecular entry point. The first approach, that of isolating novel imprinted genes, follows on from our earlier identification (Barlow *et al.*, 1991) of the mouse IGF2R/MPR300 gene as the imprinted Tme mutant gene. Information, that we are gathering on the molecular basis of imprinting this gene, is being used to devise a screen for new imprinted mouse genes. Our second approach is to isolate genes that directly regulate implantation. For this, we are attempting to positionally clone the tw73 implantation defective mouse mutant gene.

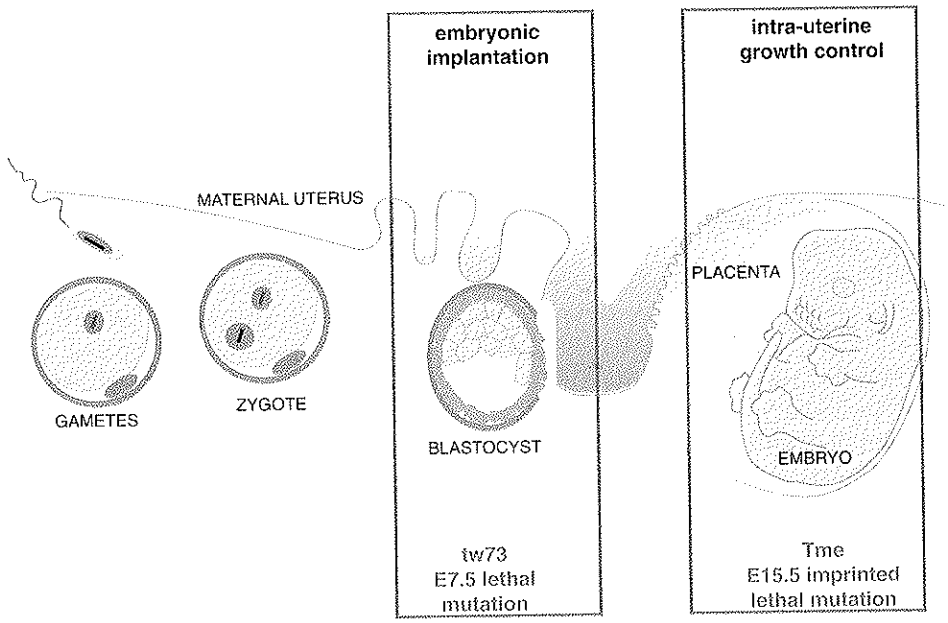

Fig.1: Mammalian embryonic development

Imprinted genes regulate embryonic growth

Mammals inherit one set of chromosomes from each parent and are therefore, with the exception of genes on the sex chromosomes, genetically diploid. A mechanism known as gametic imprinting (Barlow, 1994; Barlow, 1995) can, however, restrict expression to one parental chromosome. In the mammal, imprinted genes primarily act in embryogenesis and while there is no clear understanding why this is so, it is becoming evident that they play a fundamental role in growth control. Our work last year (Wang *et al.*, 1994) confirmed that the IGF2R/MPR300 gene also plays an important role in embryonic growth control. Thus, of the 16 known mammalian imprinted genes, 6 have been shown to play a role in embryonic growth and/or tumor growth control (see Table below).

Gene	Embryonic function	Product
WT1	lethal <E15.5	transcription factor
INS		hormone
IGF2	embryonic growth (+ve)	hormone
H19	embryonic growth (- ve)	non-ORF RNA
p57KIP2		cyclin-dep. kinase inhibitor
MASH2	placental development (+ ve)	transcription factor
SNRPN		ribonuclear protein
ZNF127		transcription factor
PAR1		
PAR5		
IPW		non-ORF RNA
IGF2R/MPR300	embryonic growth (- ve)	receptor
MAS		receptor
XIST		non-ORF RNA
PEG1/MEST		hydrolase
SP2		ribonuclear protein

Tab. 1: Imprinted genes in mammals

Functional analysis of the Imprinting Box in the mouse IGF2R/MPR300 gene locus

The mouse IGF2R/MPR300 gene is an imprinted gene, predominantly transcribed from the maternally inherited chromosome. This parental specific expression is thought to be mediated by an Imprinting Box: a 3kb CpG island located in intron 2 of the gene. Methylation of this island in the maternal germline has been proposed as the mark that acts as the parent-of-origin signal (Stöger *et al.*, 1993). Various YAC (yeast artificial chromosome) transgenes, carrying parts of the 90 kb long IGF2R/MPR300 genomic locus have been introduced into mice and their parental expression will be analyzed after germ line transmission. Two types of constructs have been engineered, using yeast homologous recombination, into these YAC constructs. One with, and one deleted for, 4 kb of the Imprinting Box sequence. The integrity of the constructs has been analyzed carefully and transgenic mice have been generated for both types of constructs. Preliminary data on the imprinted expression of these transgenes look promising and they are currently being subject to analysis.

Common characteristics of imprinted genes

Our identification of a candidate imprinting box in the mouse IGF2R/MPR300 gene prompted an analysis of other imprinted genes, to ascertain what elements these genes have in common. Our results showed that while the current set of imprinted endogenous genes and transgenes lack direct sequence homology, they do have some characteristics in common (Neumann *et al.*, 1995). Based on these analyses, we are pursuing a general strategy to isolate imprinted genes from the mouse genome that lie adjacent to methylated CG rich sequences, which contain direct repeats.

Conservation of imprinting between mouse and human

Imprinting has been shown to be conserved between mice and humans for most imprinted genes so far studied. The human IGF2R gene has been suggested to be either not imprinted or subject to a novel form of imprinting that results in a polymorphic type of monoallelic expression. We have discovered that both the methylation imprint and the putative imprinting box are, in fact, conserved in the human locus. In addition the human IGF2R locus, like all other imprinted loci, shows an apparent replication asynchrony when analyzed by flourescent in situ hybridization (Smrzka *et al.*, 1995). This suggests that the human locus, like its mouse counterpart has the capability to show monoallelic expression. We are currently investigating this using RT-PCR of human tissue. This work will be extended to examine if the frequently-occurring chromosomal changes of chromosome 6q (the location of the human IGF2R/MPR300 gene) in tumors such as human breast cancer and hepatocellular carcinoma might be associated with abnormalities of imprinting.

Positional cloning of the tw73 implantation mutant gene

The tw73 gene has been mapped by others to a small deletion, that we have shown to be approximately 550 kb long. This deletion, known as the tLub2 deletion lies on mouse chromosome 17, which by chance also contains the imprinted IGF2R/MPR300 gene. As part of a joint effort to isolate tw73, and also to characterize the extent of the imprinted domain surrounding the IGF2R/MPR300 gene, we have generated a YAC and cosmid contig spanning the tLub2 deletion. We have identified the genes that flank the IGF2R/MPR300 gene, one of which is a novel gene which has been named Lx1 (Schweifer and Barlow, submitted). Analysis of the parental allelic expression of these flanking genes has shown that neither are imprinted (Schweifer *et al.*, in preparation). Furthermore our preliminary analysis of Lx1 suggests that it is not a candidate for tw73. Work is continuing in this project to characterize other genes identified within this YAC contig.

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Molecular dissection of multistage tumorigenesis in transgenic mice

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Introduction

Several distinct genetic or epigenetic events occur during the stepwise progression of most, if not all, tumors. However, the molecular dissection of tumorigenesis is often hampered by the unavailability of tissue specimens from the multiple tumor stages. Thus, the genetic reproducibility and the accessibility of tissue specimens have made transgenic mice a valuable tool to study the molecular events involved in the stepwise progression to the tumor phenotype.

In one transgenic model of tumorigenesis (RIP1Tag2), the insulin gene regulatory region has been used to target expression of Simian Virus 40 large T antigen (Tag) to the ß cells of the pancreatic islets (Hanahan, 1985). These mice develop tumors in the ß cells of the islets of Langerhans (insulinomas) in a predictable manner, and different stages of tumor progression are clearly distinguishable (**Fig. 1**). Although all the ß cells express T antigen, only about 50 % of the 400 islets in a pancreas develop hyperplasia, as determined by increased proliferation index and increased size. Two populations of hyperplastic islets can be distinguished; the majority are in a prevascular state, whereas a small proportion (about 10 % of the total islets) secrete angiogenic factors that are chemo-attractive and mitogenic for endothelial cells. Finally, only 1 to 2 % of the islets develop into solid, highly vascularized tumors. It appears that additional genetic or epigenetic changes occur during the stepwise progression of tumorigenesis in RIP1Tag2 transgenic mice.

The major objective of our research is the identification and characterization of the molecular events that trigger the transition between the multiple stages of tumorigenesis. In particular, we investigate the molecular mechanisms that underlie tumor cell hyperproliferation, the onset of neovascularization during tumor development (tumor angiogenesis), and tumor cell invasiveness. We employ transgenic mouse models to prove causal connections between the expression of a particular gene and tumor progression *in vivo*. For example, genetic complementation of RIP1Tag2 mice with transgenic mice that overexpress a gene of interest or carry a deletion of this gene might give insight into the role these genes play in ß cell tumorigenesis in particular and in cancer development in general.

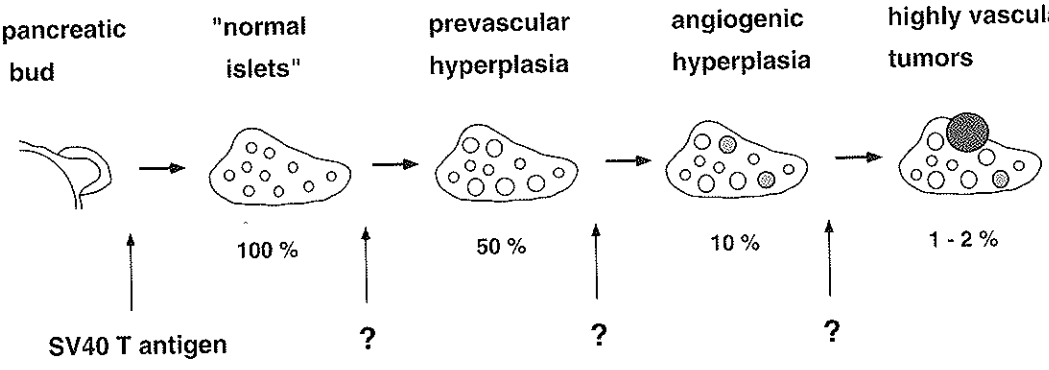

Fig. 1: Schematic representation of multistage tumor progression in RIP1Tag2 transgenic mice.

I. Tumor cell proliferation

Gabor Lamm, Rüdiger Schweigreiter, and Gerhard Christofori

We have recently reported that upregulated expression of insulin-like growth factor II (IGF-II) coincides with the onset of ß cell hyperproliferation. Indeed, IGF-II is required for the outgrowth of malignant tumors. In the absence of IGF-II, ß tumor cells continue to proliferate, yet at the same time they undergo apoptosis with dramatically increased incidence, indicating that IGF-II is a survival factor for ß tumor cells (Christofori *et al.*, 1994; Naik *et al.*, 1994). The observation that net tumor growth depends upon the balance between tumor cell proliferation and apoptosis prompted us to initiate experiments towards the molecular mechanisms that govern tumor cell survival and tumor cell death.

The up-regulation of IGF-II expression in tumor cells is not without precedent. In independent studies it has been shown that IGF-II is dramatically increased in a variety of malignancies in humans, such as breast cancer, neuroblastoma, Wilms' tumor, stomach carcinoma and others. Some cell lines derived from these cancers show growth regulation by IGF-II binding to the IGF-1 receptor (see references in Christofori and Hanahan, 1994).

I.a. Tumor cell proliferation versus tumor cell apoptosis

It is becoming increasingly apparent that the finely tuned balance between cell proliferation and **p**rogrammed **c**ell **d**eath (PCD) is a vital regulatory point during the development of malignant tumors. The requirement for the close coupling of cell proliferation and PCD during tumor development is exemplified by recent studies of RIP1Tag2 mice carrying a homozygous disruption of the IGF-II gene. In the absence of the survival factor IGF-II, tumor cells show dramatically increased incidence of PCD and a reduced tumor mass (Christofori *et al.*, 1994; Naik *et al.*, 1994; **Fig. 2**). The balance between tumor cell proliferation and tumor cell apoptosis is also apparent in cultured ß tumor cell lines. Tumor cell lines that are deficient in IGF-II expression undergo apoptosis when grown in low serum, whereas wild-type ß tumor cell lines do not exhibit a significant incidence of apoptosis when grown under these conditions. The RIP1Tag2 transgenic mice and the cultured tumor cell lines derived thereof thus provide an ideal system for studying apoptotic and survival stimuli during tumor progression. We employ both systems to functionally characterize genes that are known to play a role in the regulation of apoptosis.

For an initial analysis we focused on the ICE (**I**nterleukin-1ß **c**onverting **e**nzyme) and ICH (**I**CE and **c**ed3-like **h**omologue) family of proteases that have been reported to play a crucial role in the execution of programmed cell death. RT-PCR data on RIP1Tag2 tumors and tumor cell lines indicate that ICH splice variants but not ICE are expressed during tumor development. We would like to correlate function and expression of the ICH splice variants during tumor progression. To this end, we express inducible ICH constructs in ß tumor cell lines derived from RIP1Tag2 insulinomas. Ultimately, we plan to construct transgenic mice that carry these genes under the control of the insulin promoter and to intercross these mice with RIP1Tag2 transgenic mice. The resulting double transgenic mice will be analyzed for potential changes in ß cell tumor development. From these experiments we hope to learn about the principles that govern tumor cell survival or death *in vivo*.

I.b. IGF-II upregulation and the disruption of genomic imprinting

SV40 T antigen is expressed early during embryonic development in all ß cells in the RIP1Tag2 transgenic mice. Yet, it takes several weeks before upregulation of IGF-II expression, and with it, hyperproliferation of ß cells is first detected. To identify the link between T antigen expression and IGF-II upregulation we have intercrossed the RIP1Tag2 transgenic mice with p53-deficient mice. Surprisingly, tumor incidence and tumor mass are significantly reduced in the p53-deficient RIP1Tag2 mice. We are currently determining potential changes in tumor cell proliferation, apoptosis and IGF-II expression in the p53-deficient tumors.

It is notable that the maternal allele of the IGF-II gene is imprinted and transcriptionally repressed, whereas the paternal allele is expressed in the developing embryo. We have discovered that both alleles of IGF-II are activated during the switch to the proliferative stage in this pathway, including the imprinted maternal allele (Christofori *et al.*, 1995a). Several mechanisms could result in bi-allelic expression of IGF-II. While we have excluded the usage of different promoters between embryonic tissues and tumor cells, future experiments will have to determine whether (1) genomic imprinting is disrupted, or whether (2) the repressive imprinting signal

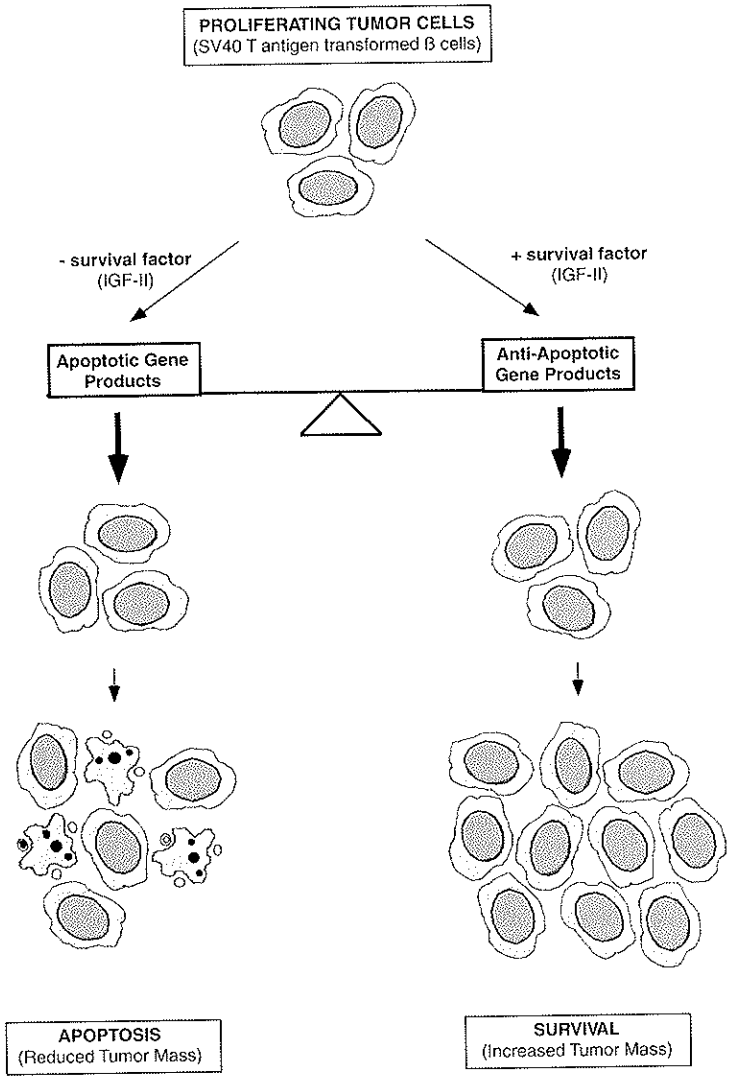

Fig. 2: The balance between tumor cell apoptosis and tumor cell survival determines the extent of tumor cell proliferation and, thus, tumor outgrowth. IGF-II provides a confirmatory signal to allow hyperproliferation of the tumor cells. In the absence of IGF-II tumor cells still proliferate, but increasingly undergo programmed cell death resulting in a net reduction of tumor mass. Whether IGF-II actively upregulates anti-apoptotic genes or whether the absence of IGF-II induces apoptotic gene function, warrants further investigation.

is ignored during transcriptional activation.

This model may therefore be of value for investigating not only regulation of the developmentally expressed and then silenced paternal allele of IGF-II, but also the mechanism of genomic imprinting and its apparent abrogation during tumorigenesis.

II. Molecular mechanisms of tumor angiogenesis

Gerhard Christofori, Amelia Compagni and Susanne Luef in collaboration with Judah Folkman and Yuen Shing (Harvard Medical School), Paul Naik and Douglas Hanahan (University of California San Francisco)

The malignant outgrowth of all solid tumors requires the formation of new blood vessels (tumor angiogenesis). Soluble growth factors that are mitogenic for endothelial cells are thought to mediate tumor angiogenesis. The first evidence for the presence of soluble factors inducing tumor angiogenesis in RIP1Tag2 transgenic mice came from *in vitro* experiments where hyperplastic islets could induce co-cultured endothelial cells to proliferate and to migrate towards the islets (Folkman *et al.*, 1989). Biochemical fractionation of medium conditioned by ß tumor cell lines and the analysis of gene expression *in vivo* indicated the presence of two known angiogenic factors: vascular endothelial growth factor (VEGF) and acidic fibroblast growth factor (FGF-1).

IIa. VEGF, tumor angiogenesis or endothelium maintainance factor?

VEGF is a multifunctional growth factor that may be responsible for the homeostasis of endocrine endothelium; it is a selective mitogen for endothelial cells and is able to permeabilize endothelium. We have analyzed the expression of VEGF mRNA and protein both in pancreatic islet cells of normal mice and in the different stages of tumor progression in RIP1Tag2 transgenic mice (Christofori *et al.*, 1995b). The 120 and the 164 amino acid isoforms of VEGF are expressed in normal islets of Langerhans and are only moderately upregulated during the different stages of tumor development. Two high-affinity receptors for VEGF, flt-1 and flk-1, are expressed by endothelial cells of normal islets, but are not upregulated during tumorigenesis.

Our data suggest that VEGF is involved in the maintenance of permeable endothelium in normal islets of Langerhans, an observation which may have implications for islet cell physiology and diabetes. VEGF may also play an important role in the growth of new blood vessels during islet cell tumorigenesis. However, since it is constitutively produced by normal islets of Langerhans, it cannot be the only factor required for the activation of tumor angiogenesis. In the future we wish to determine the physiological role of VEGF in the maintainance of permeable endothelium in endocrine organs and its involvement in tumor angiogenesis.

Cell line	Cell type	FGF synthesized	FGF exported?	FGF form*	passage number‡
βTC3	β tumor cell	FGF-1	yes	HMW	<25
βTC3	β tumor cell	FGF-1	no	-	>30
βTC111	β tumor cell	FGF-1	yes	HMW	<10
βTC69	β tumor cell	FGF-1	no	-	<10
βHC 13	β cell hyperplasia	FGF-1	no	-	<20
βHC13T	βHC13 exp. tumor§	FGF-1	yes	HMW	<10
JC10	βTC3 exp. tumor§	FGF-1	yes	HMW	>25
JB	βTC3 exp. tumor§	FGF-1	yes	HMW	>30
J39	βTC3 exp. tumor§	FGF-1	yes	HMW	>40
NF	normal dermal fibroblast	FGF-2	no	-	>20
FM14219	mild fibroma	FGF-2	no	-	>20
BPV1	fibrosarcoma	FGF-2	yes	HMW	>30
BPV3	aggressive fibroma	FGF-2	yes	HMW	>30
FM39614	mild fibroma	FGF-2	yes	HMW	>30
BPV7	aggressive fibroma	FGF-2	yes	HMW	>30
BPV11	fibrosarcoma	FGF-2	yes	HMW	>30
Balb/c 3T3	embryonic fibroblasts	FGF-2	no	-	>?
C2C12	myoblast	FGF-2	no	-	>?
HeLa	cervical carcinoma	FGF-2	no	-	>?
A459	lung carcinoma	FGF-2	no	-	>?
SKBr3	breast carcinoma	FGF-2	yes	HMW	>?
MDA MB 453	breast carcinoma	FGF-2	yes	HMW	>?
MDA MB 415	breast adenocarcinoma	FGF-2	yes	HMW	>?

* Both exported FGF-1 and FGF-2 are exclusively found in high molecular weight (HMW) forms (see text).

‡ cell lines with passage numbers above (>) or below (<) the indicated numbers have been used for the experiments.

§ 'exp. tumor' refers to tumors that have been induced by subcutaneous innoculation of the primary β cell lines βHC 13 and βTC3 into athymic mice. Secondary β tumor cell lines were then derived from these sub-cutaneous tumors.

Table 1: Many established cell lines express FGF-1 or FGF-2, but only malignant tumor cell lines export these angiogenic growth factors.

IIb. Export of FGF: a trigger for tumor angiogenesis?

Acidic and basic fibroblast growth factor (FGF-1 and FGF-2), are potent inducers of angiogenesis *in vitro* and *in vivo*. However, their involvement as paracrine factors in tumor angiogenesis has not been unequivocally demonstrated, in part because FGF-1 and FGF-2 lack classical signal sequences for secretion. The mechanism by which they are released from cells remains to be elucidated. We have demonstrated that FGF-1 is constitutively secreted by tumor cell lines derived from highly angiogenic ß cell tumors of RIP1Tag2 transgenic mice. The secreted FGF-1 is sequestered and masked in conditioned medium, as assessed by mitogenic activity and heparin affinity. High salt treatment of conditioned medium reveals FGF-1 in high molecular weight (HMW) forms with reduced heparin-affinity and a molecular mass of approximately 40 kd. Reducing agents partially release monomeric FGF-1 from the complexes, indicating that disulfide bonds are involved in the complex structure. The HMW forms of FGF-1 are found in cell lysates as well as conditioned medium, suggesting that they represent export intermediates. Brefeldin A, an inhibitor of conventional secretion, does not interfere with FGF-1 export. Similarly, FGF-2 is exported in HMW forms with reduced heparin affinity by several other tumor cell lines, including human breast carcinoma and murine fibrosarcoma, but not by normal embryo or dermal fibroblasts or by other FGF-2 expressing transformed cell lines (**Table 1**). Our data suggest that novel forms of FGF-1 and FGF-2 are selectively exported by certain tumor cell types via a non-traditional secretory pathway (Christofori *et al.*, 1996).

The high molecular weight complexes found with both FGF-1 and FGF-2 in the cell lines described above resemble each other in their biochemical properties, suggesting that both FGFs are released by a common export pathway. We plan to pursue the characterization of these complexes as an approach to elucidate the pathway of FGF release. We have also initiated experiments that are aimed at identifying the trigger for FGF export in tumor cells. Furthermore, we wish to elucidate the functional role of FGFs in tumor angiogenesis and tumor cell proliferation.

III. Changes in cell adhesion and tumor invasiveness

Anne-Karina Perl in collaboration with Henrik Semb (Umeå University, Sweden) and Harold Cremer (IBDM, Marseille)

In RIP1Tag2 transgenic mice the expression of several cell adhesion molecules changes during the outgrowth of malignant ß cell tumors. Using inducible gene expression systems in established ß tumor cell lines *in vitro* and genetic complementation of transgenic mouse lines *in vivo*, we set out to address the functional involvement of these cell adhesion molecules in tumor development. In particular, we wish to address the question whether changes in a tumor cell's adhesive repertoire are cause or consequence of tumor progression.

III.a. The role of E-Cadherin in ß cell tumorigenesis

Immunohistochemical and RNA analysis of the different stages of tumor development has revealed that the expression of E-Cadherin is downregulated in late-stage, invasive tumors which correlates with the loss of epithelial structures (**Fig. 3**). E-Cadherin expression is also very low in ß tumor cell lines that have been derived from the ß cell tumors. In preliminary experiments, stable transfection of ß tumor cell lines with E-Cadherin expression vectors resulted in markedly reduced tumorigenicity in athymic mice. Currently, we are utilizing inducible E-Cadherin expression constructs to analyze the effect of upregulated E-Cadherin expression on the phenotype of ß tumor cells, such as cell cycle arrest or the induction of apoptosis.

A more convincing test into the role of E-Cadherin during tumor development is to maintain its expression in ß cells throughout the different stages of tumor development in RIP1Tag2 transgenic mice. To this end we have produced transgenic mouse lines that express E-Cadherin under the control of the insulin promoter (RIP1E-Cad) and we are currently intercrossing these mice with RIP1Tag2 transgenic mice in order to address whether continued expression of E-Cadherin can retard or block the formation of invasive ß cell tumors *in vivo*.

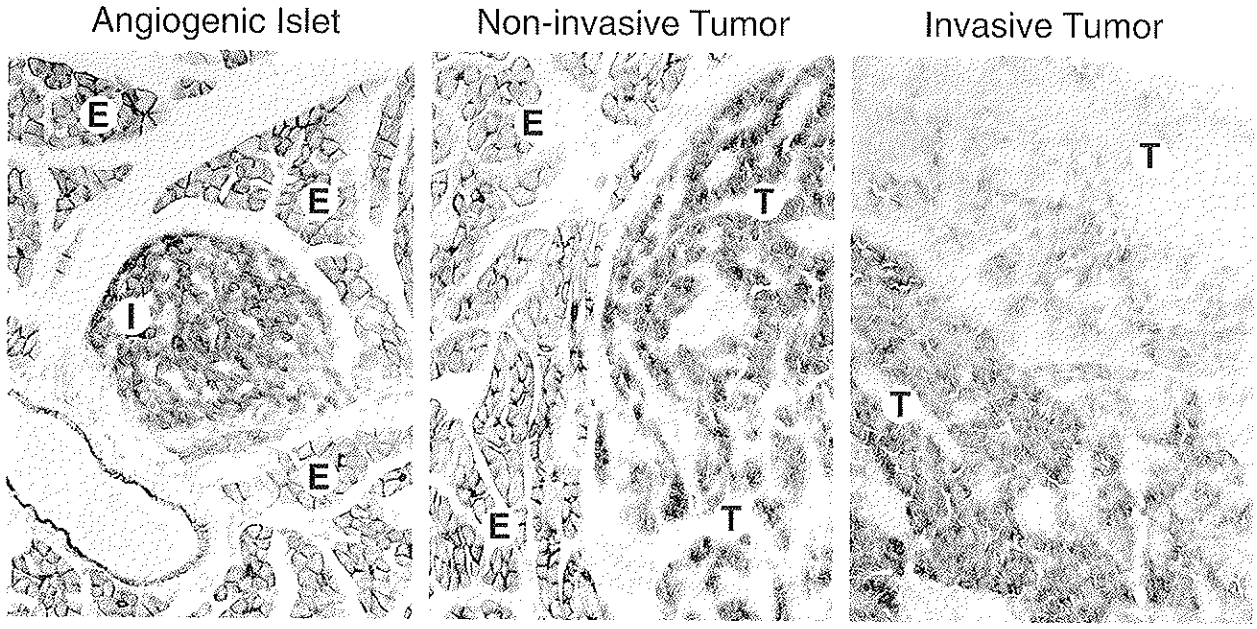

Fig. 3: Focal loss of E-Cadherin expression in late-stage ß cell tumors correlates with the loss of epithelial structures and tumor invasiveness. Histological sections of different stages of RIP1Tag2 tumor development were immunostained with an antibody specific for E-Cadherin. E-Cadherin is expressed by the acinar cells of the exocrine pancreas (E) and by the ß cells in normal islets, hyperplastic islets and angiogenic islets (left panel; I) and is unchanged in the epithelial structures of ß cells in non-invasive tumors (middle panel; T). However, E-Cadherin expression is downregulated in the invasive parts of late-stage tumors correlating with the loss of epithelial structures (upper half of the right panel; T).

III.b. Switch in N-CAM isoforms during ß cell tumorigenesis

Our studies have also revealed that the expression of neuronal cell adhesion molecule (N-CAM) changes in the later stages of ß cell tumor development. The 120 kd GPI-linked isoform on the surface of normal ß cells is almost completely replaced by the highly polysialylated 140 and 180 kd transmembrane isoforms in ß tumor cells. A similar change in N-CAM expression has been described in the development of Wilms' tumor (Roth *et al.*, 1988).

We wish to define the functional role of N-CAM in ß cell tumor development. Inactivation of the N-CAM gene by homologous recombination resulted in mice with relatively minor phenotypic changes such as reduction of the olfactory bulb and deficits in spatial learning (Cremer *et al.*, 1994). The N-CAM deficient mice are currently being employed to produce RIP1Tag2 transgenic mice that lack any functional N-CAM allele, and we will examine these mice for phenotypic changes in ß cell tumor development. It is possible, however, that the complete absence of N-CAM expression has no direct effect on ß cell tumor development, since the switch from the 120 kd isoform in normal ß cells to the 140 and 180 kd forms in tumors might result in a functional downregulation of N-CAM-mediated cell-cell-adhesion. We are addressing this question by constructing transgenic mouse lines that overexpress the 120 kd isoform of N-CAM in the ß cells of the islets of Langerhans (RIP N-CAM120). We will then produce double transgenic RIP1Tag2 x RIP N-CAM120 mice and determine potential changes in their tumor phenotype.

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Virus entry mechanisms and gene delivery

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Overview, main research topics

We are studying in detail the process and the consequences of adenovirus entry into eukaryotic cells. Our strategy is to develop a well-founded understanding of adenovirus and use this to improve the vectors used for gene delivery. We have attempted to strike a balance between basic research (determining how adenovirus functions) and applied research (determining how the current adenovirus gene delivery systems function and how they can be improved). There is very little information available on the mechanisms used by any virus to enter its eukaryotic host cell and we hope to define the process used by adenovirus. The subgroup C adenoviruses can initiate infection from a single particle, demonstrating that the entry functions of this virus are very effective. The strategies that adenovirus has evolved to deposit its nucleic acids within cells may also be used in the design of synthetic DNA delivery systems. In addition, we have found that the entry of adenovirus triggers a series of important host responses including the activation of transcription factors involved in inflammatory responses (e.g. NF-κB) and the initiation of apoptotic responses. A clear analysis of the events following adenovirus entry will provide information about both apoptosis and inflammation.

Several years have passed since transferrinfection was developed as a method of using receptor-mediated endocytosis to delivery DNA (reviewed in Cotten and Wagner, 1993; Cotten, 1995). The currently used transferrinfection system (developed with the groups of Max Birnstiel and Ernst Wagner) is briefly described in **Figure 1** (see the **Fig. 1** legend for details). A crucial component of the system is a psoralen-inactivated adenovirus particle (Cotten *et al.*, 1992; Cotten *et al.*, 1994a) which serves a carrier function and aids the passage of polylysine-condensed DNA through the target cell membrane, cytoplasm and into the nucleus. Genes to be delivered into cells are carried as polylysine condensed bundles attached to the carrier virus.

Adenovirus-based gene delivery systems are the most efficient vectors for gene delivery available to date. No other virus system can survive the bloodstream or can function efficiently in intra-tumoral applications. However, adenovirus vectors provoke a potent humoral and cellular immune response that limits their use for long-term gene correction but promotes their function in vaccine applications. We have attempted to understand why adenovirus is such an antagonistic vector.

The work in our group over the past year has identified several phenomena associated with adenovirus and its host cell entry that are important for virology and may be relevant to gene therapy :

i. Adenovirus entry in the complete absence of virus gene expression activates an inflammatory transcription program.

ii. Adenovirus-mediated transferrinfection activates an apoptotic response that can be blocked by anti-inflammatory drugs or by anti-apoptotic gene expression.

iii. The adenovirus protease is directly required during virus entry and may be responsible in part or solely for the cellular inflammatory response to virus entry.

In addition, we have initiated a more thorough characterization of the avian adenovirus CELO. In the past we demonstrated that this virus possessed similar gene delivery properties as the mastadenoviruses normally used for gene delivery (Cotten *et al.*, 1993). Further analysis of CELO resulted in the identification of a novel anti-apoptotic gene carried by the virus. Additional analysis of the CELO virus genome revealed a number of additional unique features of this adenovirus which may make this virus ideally suited for vector use in gene therapy.

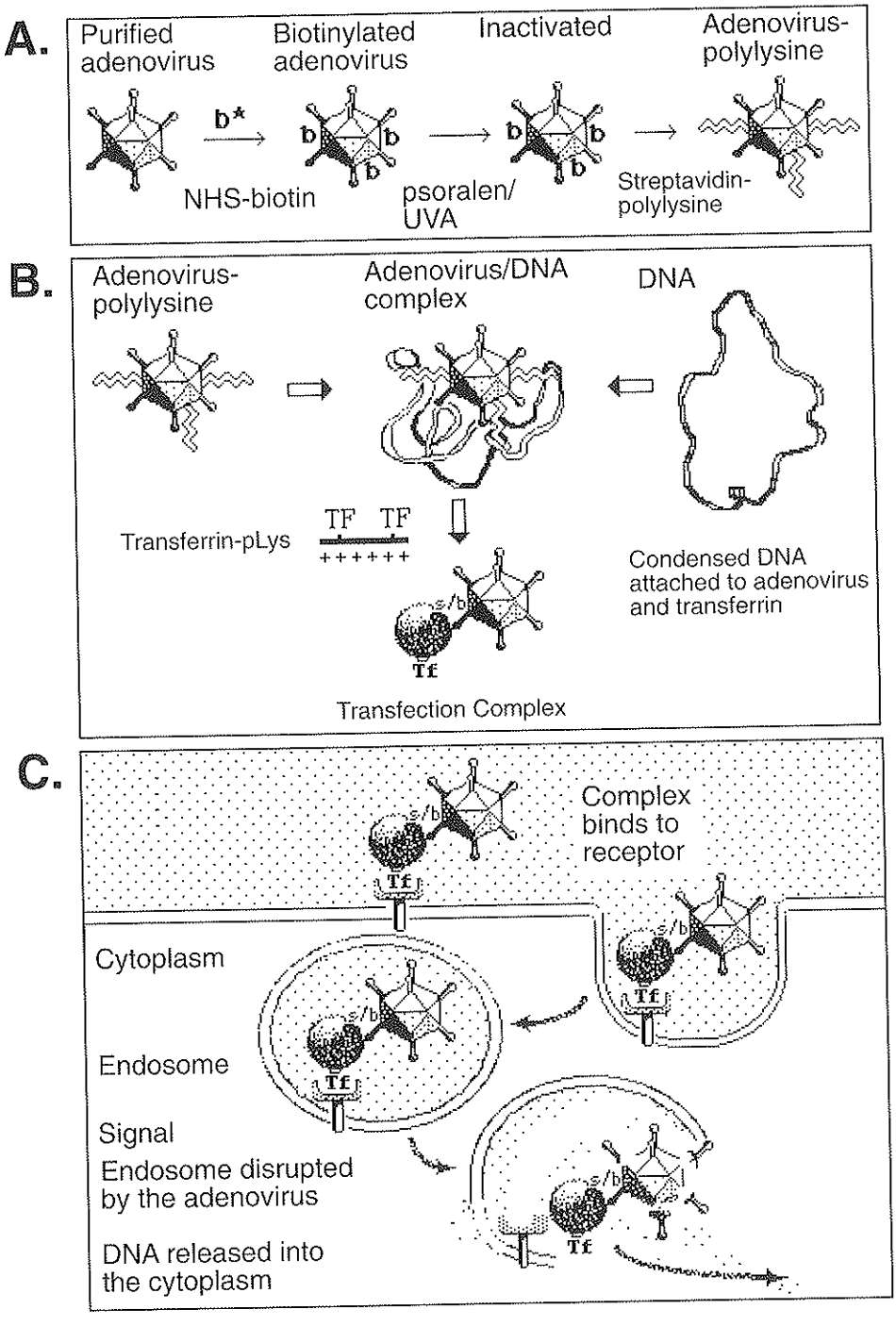


Fig. 1a: Biotinylation and UV inactivation of adenovirus. Exposed lysine residues on the surface of adenovirus particles are modifed with biotin groups. The biotinylated virus is then treated with the DNA intercalator 8-methoxypsoralen and irradiated with 360 nm UV light to destroy the viral DNA (Cotten *et al.*, 1994). DNA binding polylysine is then attached to the exterior of the virus using streptavidin-polylysine (Wagner *et al.*, 1992).

Fig. 1b: Assembly of adenovirus/ polylysine/DNA transfection complexes. Adenovirus polylysine is mixed with DNA containing the gene to be delivered. Polylysine/ DNA interactions link the DNA to the virus. Full condensation of the DNA and attachment of a cell-binding ligand (e.g. transferrin) are obtained by the addition of transferrin-polylysine to generate the transfection complex.

Fig. 1c: DNA delivery. Transfection complexes are supplied to target cells. Interactions with the cell receptors for transferrin, the virus fiber and the penton base (as well as non-specific polylysine/cell interactions) result in internalization of the transfection complex. Signals generated by the internalization of the endosome (e.g. decreased pH) activate the membrane disruption activity of the virus and allow the virus (and associated polylysine/DNA) to enter the target cell cytoplasm and nucleus.

Adenovirus entry induces both inflammatory and apoptotic responses that enhance vaccine applications but interfere with long-term cell viability.

Adam Baker, Susanna Chiocca and Matt Cotten

Adenovirus entry into eukaryotic cells can activate a series of responses that may interfere with long-term survival of the modified cells but may also enhance the immune response to these cells in a vaccine application. These cellular responses to adenovirus entry are summarized in **Figure 2.**

We have shown that cell death is triggered by the entry of the adenovirus, even in the absence of viral gene expression. In an attempt to determine if adenovirus gene expression could influence this host

response we systematically included various adenoviral genes in the transfection complexes and identified the E1B 19K as the protective gene. It is known that the E1B 19K protein can function as a potent analogue of the apoptosis-blocking host gene Bcl-2. Expression of either E1B 19K or Bcl-2 in transferrin-fection systems enhanced gene expression over time.

We have now modified our standard cotransfection assay in order to specifically monitor the fate of transfected cells. The assay uses the green fluorescent protein (GFP) as a reporter gene in place of luciferase. Transfected cells expressing GFP emit an intense fluorescence that allows us to follow the morphology of the transfected cells. GFP-expressing cells in control transfections show an apoptotic phenotype at 5 and 8 days post-transfection which is blocked in cells expressing Bcl-2 or E1B 19K.

We have identified an inflammatory response which compromises the health of the transfected cells at later times. We have shown that adenovirus entry up-regulates at least two inflammatory gene promoters: an NF-κB dependent artificial promoter and the NF-κB dependent IL-6 promoter. The activation occurs in the absence of viral gene expression, indicating that a virion component or the entry process itself causes the inflammatory response. Using transient transfections, stable cell lines and electrophoretic mobility shifts, we have shown that NF-κB and NF-IL6 are activated in response to adenovirus entry. Anti-inflammatory drugs, such as the glucocorticoid dexamethasone, block the inflammatory response to the adenovirus and dramatically enhance gene expression over time. These modifications have been incorporated into our efforts to express Factor VIII to ameliorate hemophilia A. Previous attempts in primary cells allowed factor VIII expression for less than 48 hours (Zatloukal *et al.*, 1994). Including anti-inflammatory agents prolongs expression to at least 21 days.

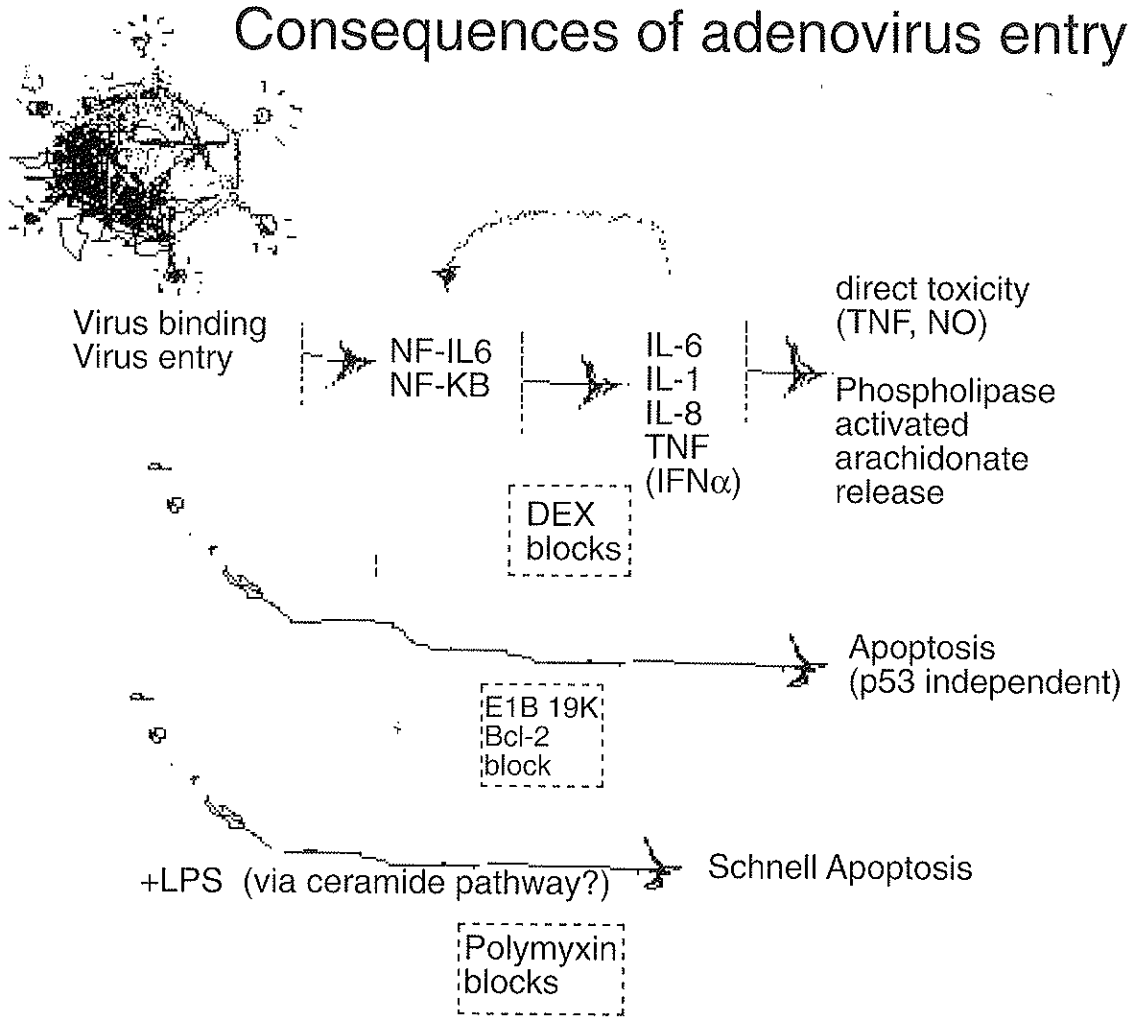


Fig. 2: The consequences of adenovirus entry. The binding and entry of adenovirus particles can activate an apoptotic response (blocked by E1B 19K, Bcl-2 or GAM-1) and an inflammatory response (blocked by glucocorticoids such as dexamethasone). In the presence of lipopolysaccharide (LPS, a common contaminant of DNA and our environment) a rapid apoptotic response can occur. This can be blocked by careful preparation of DNA (Cotten *et al.*, 1994) or by including the LPS-binding antibiotic polymyxin in the transfection medium.

The identification and characterization of GAM-1, a novel anti-apoptotic gene from the chicken adenovirus CELO.

Susanna Chiocca and Adam Baker

We have demonstrated that the decline in cell viability induced by adenovirus-augmented transfection in primary human fibroblasts can be prevented by expression of the anti-apoptotic genes E1B 19K or Bcl-2. This forms the basis of a simple screen for novel anti-apoptotic genes: co-transfect a luciferase expression plasmid with a test gene and look for an enhancement in long-term gene expression. Our experience with the avian adenovirus type 1 (CELO virus) suggested that the virus must somehow increase the survival of transfected cells (Cotten *et al.*, 1993). We therefore screened for anti-apoptotic genes in the CELO virus and identified a protective genome region. Sequencing and deletion analysis of this region has identified a single gene (named GAM-1 for **G**allus **a**nti **m**orte) with no homologies to the known anti-apoptotic genes.

GAM-1 mimics Bcl-2 and E1B-19K in a number of survival assays. Mutational analysis of GAM-1 has identified a leucine zipper motif that suggests a site for either homo-dimerization or for interaction with other proteins; alteration or removal of this region from the protein eliminates the antiapoptotic function. Future work will identify the binding partners for GAM-1. Because GAM-1 lacks the standard protein binding sequences found in all members of the Bcl-2 family (the BH1 and BH2 motifs), it is possible that identifying GAM-1 partners will identify novel participants in the apoptotic pathway. Because there are now links between inflammation, the interferon response, apoptosis and oncogenesis, identifying the components of this apoptotic pathway can identify important clinical targets.

The complete DNA sequence and genomic organization of the chicken adenovirus CELO.

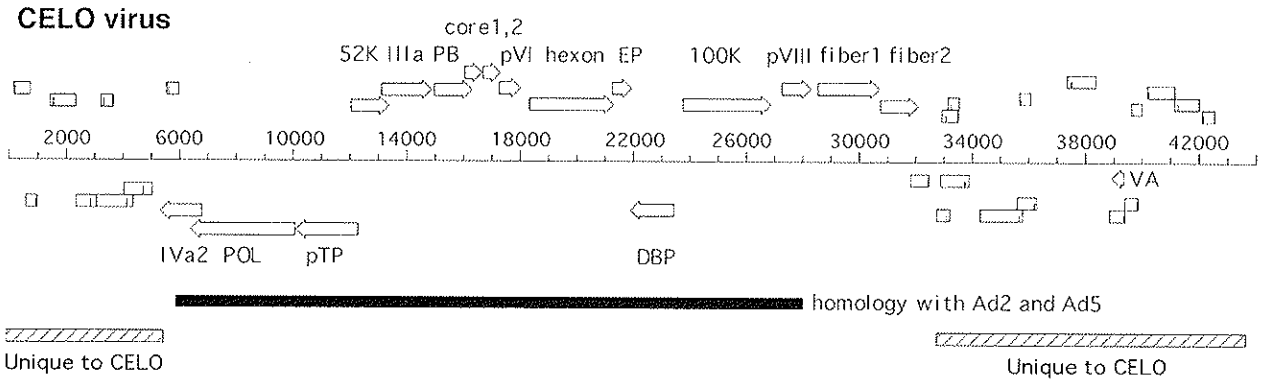
Susanna Chiocca, Robert Kurzbauer, Gotthold Schaffner, Adam Baker and Matt Cotten (IMP) and Vivien Mautner, MRC Virology Unit, University of Glasgow

Our interest in the avian adenovirus CELO (chicken embryo lethal orphan) stems from our work demonstrating that this virus possessed useful gene delivery activity (Cotten *et al.*, 1993). Our efforts to identify an anti-apoptotic gene were successful (see above) and also demonstrated that CELO lacked the standard E1 gene cluster found in all other adenoviruses. The complete sequence of the CELO genome (Chiocca *et al.*, 1996; see **Fig. 3**) reveals a number of important differences between CELO and the other adenoviruses.

1. The CELO genome. The CELO genome is 43.8 kb, 8 kb longer than the human subgenus C adenoviruses (Ad2 and Ad5). The genes for major viral structural proteins and the early region 2 genes are present in the expected locations in the genome. However, the CELO virus lacks sequences homologous to the mammalian adenovirus E1, E3 and E4 regions. There are 15 kb of new sequence at the ends of the CELO genome, rich in open reading frames, and it is likely that these encode functions that replace the missing E1, E3 and E4 regions.

2. Transformation. Like many of the human adenoviruses, CELO can transform some cell types and will induce tumors in baby hamsters. However, CELO has no E1 genes, which are solely responsible for the transforming and oncogenic potential of the human adenoviruses. Ad5 transformation requires growth stimulating functions (provided by E1A) that solve the RB/E2F problems and anti-apoptotic functions (provided by E1B 19K or E1B 55K) which solve the p53 and apoptosis problems. We find that like Ad5, transformation with CELO requires at least two genes, one of which is the anti-apoptotic gene GAM-1. Identification of the CELO gene product that interacts with RB is of interest because the manner in which CELO solves this problem can give information about both RB and CELO. This identification is also essential for vector development because these viral genes must be deleted.

3. Vector development. CELO is naturally defective in mammalian cells. Vectors based on CELO can be used in humans with no risk that a subsequent infection with a wild-type human adenovirus will complement the vector and allow replication. This is not the case with the Ad5-based vectors currently in clinical use. The CELO genome is 8 kb longer than the Ad5 genome. Both viruses have comparable virion dimensions, so the CELO has a method of relieving the strict 35 kb packaging limit found in Ad5 and this increased genome capacity will be important for vectors carrying large inserts. CELO does not bind human cells which will allow us to introduce targeting modifications in the CELO virion.



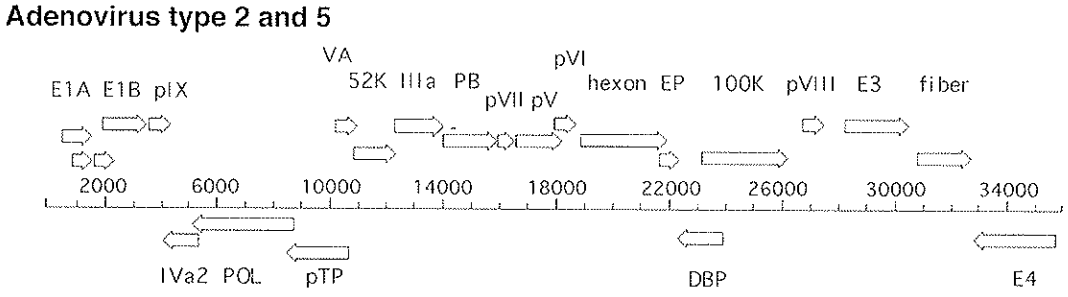


Fig. 3: Comparison of Ad2/5 vs CELO virus genomic organization. A summary of the Ad2/5 and CELO virus genomic organization is presented (Chiocca *et al.*, 1996). The arrows indicate the locations of coding regions but not the precise splicing patterns of the gene products. No indication of reading frame is implied by the positions of the ORF arrows. The CELO virus pattern also indicates (in the first 6000 bp and the last 13000 bp) all unassigned open reading frames commencing with a methionine and encoding >99 amino acid residues. The central region of the two genomes that show homology by dot matrix analysis as well as regions at the ends of the CELO virus genome with no homology to other adenoviruses (Unique to CELO) are indicated. Abbreviations: PB, penton base; EP, endoproteinase; DBP, DNA binding protein; pTP, pre-terminal protein; pol, DNA polymerase.

The role of the adenovirus protease in virus entry and in the inflammatory response that accompanies virus entry

Birgit Panzenböck, Adam Baker, Heike Lehrmann and Matt Cotten

Adenovirus encodes a 23 kilodalton cysteine protease which is essential in three fundamental stages of the viral life cycle:

1. Limited proteolysis of six virus structural proteins, pVI, pVII, pVIII, IIIa, preterminal protein and the 11K protein by the protease is required for the production of mature, infectious adenovirus particles.

2. Late in infection, the protease cleaves host cytoskeletal proteins (e.g. cytokeratin 18), weakening the host cell and allowing release of progeny virions.

3. Protease carried by the virion functions during the infectious entry of adenovirus either to cleave viral proteins to promote the dismantling of the virion, or to cleave host proteins to facilitate virus entry (Cotten and Weber, 1995).

We found that co-expression of a recombinant protease gene results in an initial upregulation of several NF-κB dependent promoters followed by a sharp decline in expression consistent with cell death. The decline is blocked by the expression of E1B 19K. Furthermore, direct analysis of protease-expressing cells using the green fluorescent protein reveals an apoptotic morphology induced by protease expression. The initial induction of gene expression and the later induction of apoptosis may be important functions of the adenovirus protease during the viral life cycle. Analyses of these phenomena are underway in an effort to understand if efforts to limit adenovirus protease action can prevent the inflammatory responses to virus entry.

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Mammalian chromatin regulators and gene activity during mouse development

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Postdoc	Götz LAIBLE
PhD student	Andrea WOLF
Diploma student	Babak HARIRI (since Nov. 95)
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Summer student	Dan POPKIN (Jun.-Jul. 95)
Part-time student	Philipp SELENKO (Sept.-Oct. 95)

Introduction

The organization of DNA into distinct chromosomal domains represents a crucial mechanism to govern the activity of key regulator loci in eukaryotes. Paradigms for chromatin-controlled maintenance of gene expression patterns include *polycomb*-dependent (*Pc*-G) repression of homeotic selector genes in *Drosophila* and *SIR*-dependent mating type switching in *S. cerevisiae*. Our research is focused (i) on the isolation and biochemical characterization of mammalian homologues of *Drosophila* and yeast chromatin regulators and (ii) on the analysis of the *in vivo* function of mammalian chromatin regulators during mouse development by generating gain- and loss-of-function mutations.

I. Mammalian homologues of *Drosophila* chromatin regulators

In *Drosophila*, both activators (*trithorax* or *trx*-group) and repressors (*polycomb* or *Pc*-group) of chromatin-dependent gene activity within the homeotic gene cluster have been described. The few known mammalian homologues, like human *HRX* (*trx*-group) (Tkachuk *et al.*, 1992; Gu *et al.*, 1992; Yu *et al.*, 1995) and murine *bmi-1* (Pc-group) (van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991; van der Lugt *et al.*, 1994) seem to serve similar functions, since mutated alleles disrupt normal cell proliferation (both are involved in the generation of B-cell leukemia) and cause homeotic transformations (Tkachuk *et al.*, 1992; Gu *et al.*, 1992; Yu *et al.*, 1995; van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991; van der Lugt *et al.*, 1994). Recently, a 130 amino acid carboxy-terminal region of sequence similarity has been identified that is shared between a positive (***trx***, trx-group) and a negative (***E(z)***, *Pc-group) Drosophila chromatin regulator* (Jones and Gelbart., 1993). Furthermore, this carboxy terminus is also conserved in ***Su(var)3-9***, a dominant suppressor of position-effect-variegation (PEV) and heterochromatin spreading in *Drosophila* (Tschiersch *et al.*, 1994). It has been suggested that this domain may play a role in the assembly of either an activating or a repressing chromatin structure, dependent on interacting accessory proteins from the *trx*- or *Pc*-group. Because of its evolutionary conservation and the presence in antagonistic gene products, this novel protein domain, designated SET (Tschiersch *et al.*, 1994), may define a new gene family of developmentally important chromatin regulators.

Functional analysis of mammalian cDNAs encoding the conserved SET domain of the *Drosophila* chromatin regulators *E(z)* and *Su(var)3-9*

Götz Laible and Angelika Lebersorger, in collaboration with Rainer Dorn and Gunter Reuter (University of Halle, Germany)

Based on sequence information from the conserved SET domain, we isolated from human and mouse cDNA libraries several clones that are the mammalian homologues of *Drosophila E(z)* and *Su(var)3-9* (see **Fig. 1**). The overall amino acid identities between the *Drosophila* and human proteins are 61% for *E(z)*/*EZH2* and 43% for *Su(var)3-9*/*SUV39H*, with the carboxy-terminal SET domain being very highly conserved (88% for *EZH2* and 55% for *SUV39H*). Sequence comparison revealed additional distinct regions of homology, like a cystein-rich domain in *EZH2* and a chromo-box in *SUV39H*. By

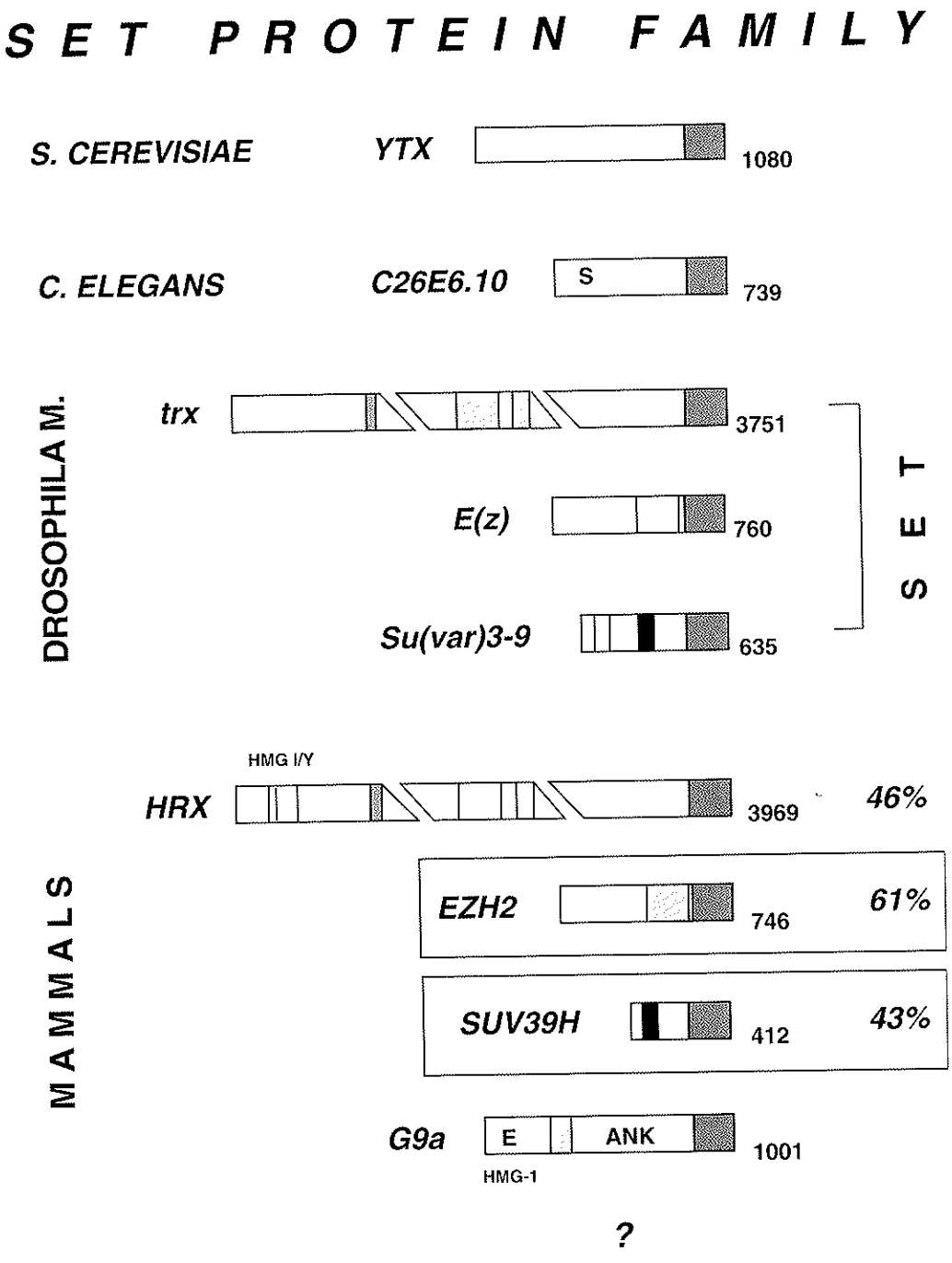
SET PROTEIN FAMILY
S. CEREVISIAE
YTX
1080
C. ELEGANS
C26E6.10
S
739
DROSOPHILA M.
trx
3751
E(z)
760
Su(var)3-9
635
SET
HMG I/Y
HRX
3969
46%
MAMMALS
EZH2
746
61%
SUV39H
412
43%
G9a
E
ANK
1001
HMG-1
?

Figure 1

contrast, 206 amino acids comprising an amino-terminal GTP binding motif present in the fly protein are missing in human *SUV39H*. Southern analyses indicated that *Su(var)3-9* homologues are single genes in mouse and man, whereas *E(z)* sequences are encoded by two distinct loci.

Using mouse-specific RNA probes, we analyzed the expression profiles of murine *Ezh1* and *Suv39h* by RNAse protection. Both genes are widely expressed, however differ in their relative abundance during mouse development. Whereas *Ezh1* is up-regulated approximately four- to five-fold in several mature tissues as compared to embryonic tissues, *Suv39h* is expressed at reproducibly higher levels during early embryonic development but downregulated at later stages. These data suggest different roles for *Ezh1* and *Suv39h* during mammalian development. Furthermore, the differences in the expression levels between embryonic and mature tissues are reminiscent of gene dosage effects observed with several *Drosophila* chromatin regulators.

To demonstrate involvement of *EZH2* and *SUV39H* in chromatin-controlled gene regulation, we used position-effect-variegation (PEV) of a heterochromatin-associated *white* gene in the *Drosophila* marker strain *In(1)w^{m4}*. In its normal chromosomal position, the *white* gene specifies red eye color but as a consequence of the *In(1)w^{m4}* inversion is misplaced adjacent to heterochromatin, resulting in variable gene activities that can be easily visualized as red and white patches in the *Drosophila* eye. In collaboration with Rainer Dorn and Gunter Reuter (University of Halle, Germany) transgenic flies were generated that overexpress our human cDNAs from an inducible heat-shock promoter. Heat-shocked flies, transgenic for either *EZH2* or *SUV39H*, exhibit a marked suppression of the *In(1)w^{m4}* marker gene that can be detected as enlarged white patches in the *Drosophila* eye. These data indicate that both of our mammalian cDNAs encode functionally conserved gene products that participate in the regulation of chromatin-dependent gene activity.

Biochemical characterization of mammalian protein complexes containing *EZH2/Ezh1* and *SUV39H/Suv39h* epitopes

Götz Laible, Babak Hariri and Philipp Selenko

In *Drosophila*, genetic data and co-localization studies of immunostained polytene chromosomes suggest that chromatin regulators are organized in multimeric protein complexes (Messmer *et al.*, 1992; Platero *et al.*, 1995). To address directly whether our mammalian cDNAs encode gene products that can physically interact, we co-translated epitope-tagged *EZH2* and *SUV39H in vitro*. Following immunoprecipitation with epitope-specific antibodies, we demonstrate homo-dimerization of *SUV39H* and (weak) complex formation between *SUV39H* and *EZH2*. Using transient transfection of fibroblasts, we are currently extending these *in vitro* studies to investigate whether epitope-tagged *SUV39H* and *EZH2* co-immunoprecipitate endo-genous proteins *in vivo*.

Recently, the chromo-boxes of *polycomb* and *HP1* were shown to be required for target-specific chromatin association and protein-protein interaction (Messmer *et al.*, 1992; Platero *et al.*, 1995). To investigate whether the chromo-box and/or SET domains of *SUV39H* and *EZH2* are involved in mediating protein-protein interaction, truncated products comprising the chromo-box (*SUV39H*) or the SET domains (*SUV39H* and *EZH2*) were generated from *in vitro* translated cDNAs and tested for interaction with a variety of heterologous, *in vitro* co-translated chromatin regulators containing similar protein domains (e.g. *Drosophila HP1*, *E(z)*, *Su(var)3-9* and yeast *YTX*; see **Fig. 1**). These studies indicated that the chromo-box of *SUV39H* is a crucial determinant to mediate protein-protein interactions *in vitro*.

In order to analyze endogenous protein complexes containing murine *Ezh1* and *Suv39h* epitopes *in vivo*, glutathion-S-transferase (GST) fusion proteins encoding *Suv39h* or *Ezh1* sequences were expressed and are being purified from bacteria. Together with several synthetic peptides, these fusion proteins will be used to raise polyclonal antibodies for the biochemical purification of endogenous protein complexes containing *Ezh1* and *Suv39h* epitopes and for the immunolocalization of putative target sequences in chromatin.

Gain- and loss-of-function of *EZH2/Ezh1* and *SUV39H/Suv39h* in the mouse

Thomas Jenuwein, Angelika Lebersorger, Dan Popkin and Götz Laible

The proto-oncogene *bmi-1* is the first mammalian homologue of a *Drosophila* chromatin regulator whose function has been analyzed in the mouse (van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991; van der Lugt *et al.*, 1994). Interestingly, overexpression of a *bmi-1* transgene resulted in dose-dependent anterior transformations of the axial skeleton and induced leukemia (Alkema *et al.*, 1995). Similar to *bmi-1*, *Ezh1* and *Suv39h* are widely expressed during mouse development. Moreover, *Ezh1* and *bmi-1* are both

members of the *Pc*-group of genes. We are using the same transgenic *bmi-1* vector (Alkema *et al.*, 1995) to drive overexpression of epitope-tagged *EZH2* and *SUV39H* sequences in the mouse. To facilitate expression of the cDNAs, we included a β-globin intron at the 5' end of the cDNAs.

For each of the two gene constructs, a total of ten founder animals were generated. As expected, transmission of the transgenes varied among the founder animals and, out of eight transgenic lines analyzed so far for the *SUV39H* construct, three mouse lines exhibited a five- to ten-fold overexpression of the transgene. Taking the specific cartilage protrusions of the cervical C6 and thoracic T2 vertebrae as references, we observe posterior transformations (C7—>T1 and T1—>T2) of the axial skeleton of F_1 fetuses (see **Fig. 2**) that persist along the antero-posterior axis. In addition, a duplicated and partially fused R1 rib is present asymmetrically. These data indicate that the mammalian homologue of *Su(var)3-9* is involved in specifying the antero-posterior axis, most likely by regulating the expression boundaries of the murine *HOX* gene clusters (Alkema *et al.*, 1995).

In order to generate loss-of-function mutations, we have isolated mouse genomic clones encoding *Ezh1* (C57Bl6 cosmid library) and *Suv39h* (129 λ library) sequences. The characterization of the genomic clones demonstrated a complex exon/intron structure of the murine *Ezh1* locus and confirmed the 206 amino acid, amino-terminal truncation of the mammalian *Su(var)3-9* homologue, because sequence analysis predicted a GC-rich consensus promoter that precedes the 5' end of our cDNAs. Currently, we are in the process of generating ES cell clones in which the endogenous *Ezh1* and *Suv39h* loci have been disrupted by homologous recombination.

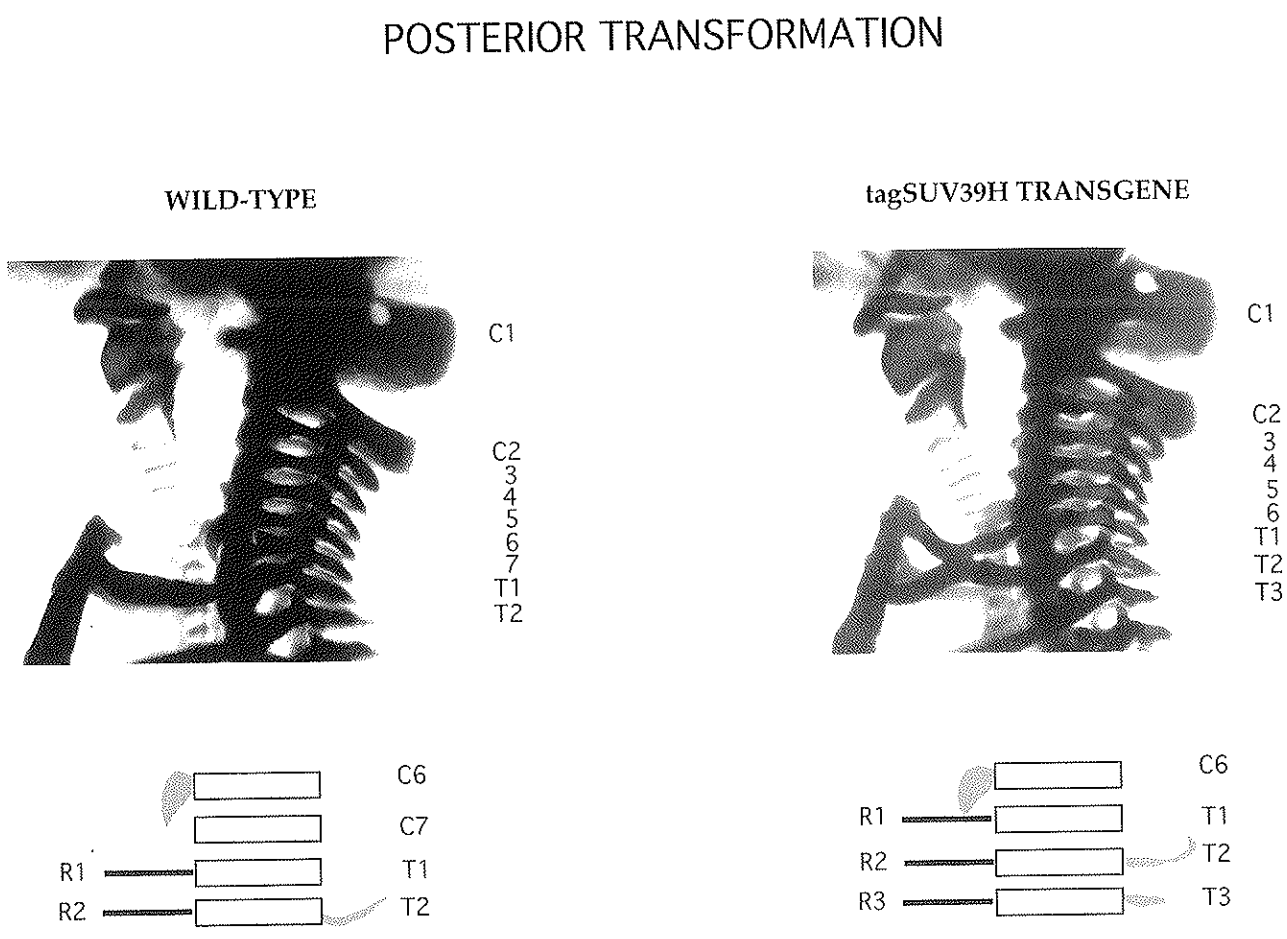

Figure 2

II. Mammalian homologues of *S. cerevisiae* chromatin regulators

Although chromatin in *S. cerevisiae* is less compacted than that of higher eukaryotes, the underlying mechanisms that restrict gene expression at yeast telomeres and at the silent mating type loci bear striking similarities to processes involved in PEV in *Drosophila* and X-inactivation in mammals. *SIR* genes (for **s**ilent **i**nformation **r**egulator) have been shown to be crucial components of heterochromatin-like complexes at telomeres and silent mating type loci in yeast. Furthermore, mutations in *SIR4* cause aberrant gene expression leading to perturbed mating (no cell type switching) and extended life span (delayed aging) (Kennedy *et al.*, 1995). Given the important function of *SIR* genes during the yeast life cycle, homologues of *SIR* genes are extremely attractive candidates to regulate chromatin-dependent developmental changes in gene expression patterns in higher eukaryotes. We started to exploit interference of telomeric silencing in yeast marker strains (Renauld *et al.*, 1993; Singer and Gottschling, 1994) as a screen for mammalian homologues of *SIR* genes.

Interference of telomeric silencing as a screen for mammalian homologues of the yeast SIR4 gene

Andrea Wolf

Dependent on gene dosage, *SIR* proteins will assemble or interfere with the establishment of a repressive chromatin structure at telomeric positions. Yeast strains that contain telomeric *URA* and telomeric *ADE* markers fail to grow in medium lacking uracil and form red/white sectored, variegating colonies in complete medium (see **Fig. 3**). Overexpression of *SIR4* leads to a redistribution of silencing complexes (interference) and relieves gene repression at telomeric positions (Renauld *et al.*, 1993; Singer and Gottschling, 1994). Therefore, yeast cells with an increased *SIR4* dosage form white colonies on ura$^-$ plates. We are exploiting this double selection system of interference of telomeric silencing to screen a human cDNA library in a gal-inducible yeast expression vector (gift of S. Elledge, University of Houston, Texas) for mammalian homologues of *SIR4*. In our first round of screening, we obtained approximately 60 double positive colonies (ura$^+$, white). Three out of these 60 colonies reverted back to wild-type upon plasmid loss, indicating a high percentage of suppressor mutations. The characterization/plasmid-dependence of these positive clones is currently under investigation.

Functional conservation of chromatin regulators from yeast to man

Andrea Wolf, in collaboration with Corey Nislow and Lorraine Pillus (University of Boulder, Colorado)

Genes that contain a chromo-box or a SET domain have recently been identified through the yeast genome project (see also **Fig. 1**), suggesting a functional conservation of chromatin regulators during evolution. Thus, we became interested in analyzing the potential of mammalian *EZH2* and *SUV39H* gene products in interfering with telomeric silencing in yeast. Overexpression of epitope-tagged, full-length and truncated versions (chromo-box and SET-domains) of *EZH2* and *SUV39H* in the same telomeric marker strains that are used for the *SIR4* screen (see above) indicated no significant depression (interference) of telomeric markers. However, as demonstrated for a *GAL4-SIR1* fusion protein (Renauld *et al.*, 1993), tethering of chromatin regulators to DNA may be required to nucleate interference. Using a yeast strain that contains *GAL4* binding sites in front of a telomeric *URA* marker gene (Chien *et al.*, 1993), we targeted a *GAL4-SUV39H^{chromo}* fusion protein to this modified telomere. Overexpression of the *GAL4-SUV39H^{chromo}* fusion protein resulted in partial suppression of telomeric silencing. Together with the recently published EST-sequences of putative mammalian *SIR2* homologues (Brachmann *et al.*, 1995), these data provide the first experimental evidence for a functional conservation between mammalian and yeast chromatin regulators.

INTERFERENCE WITH TELOMERIC SILENCING AS A SCREEN FOR MAMMALIAN SIR4 HOMOLOGUES

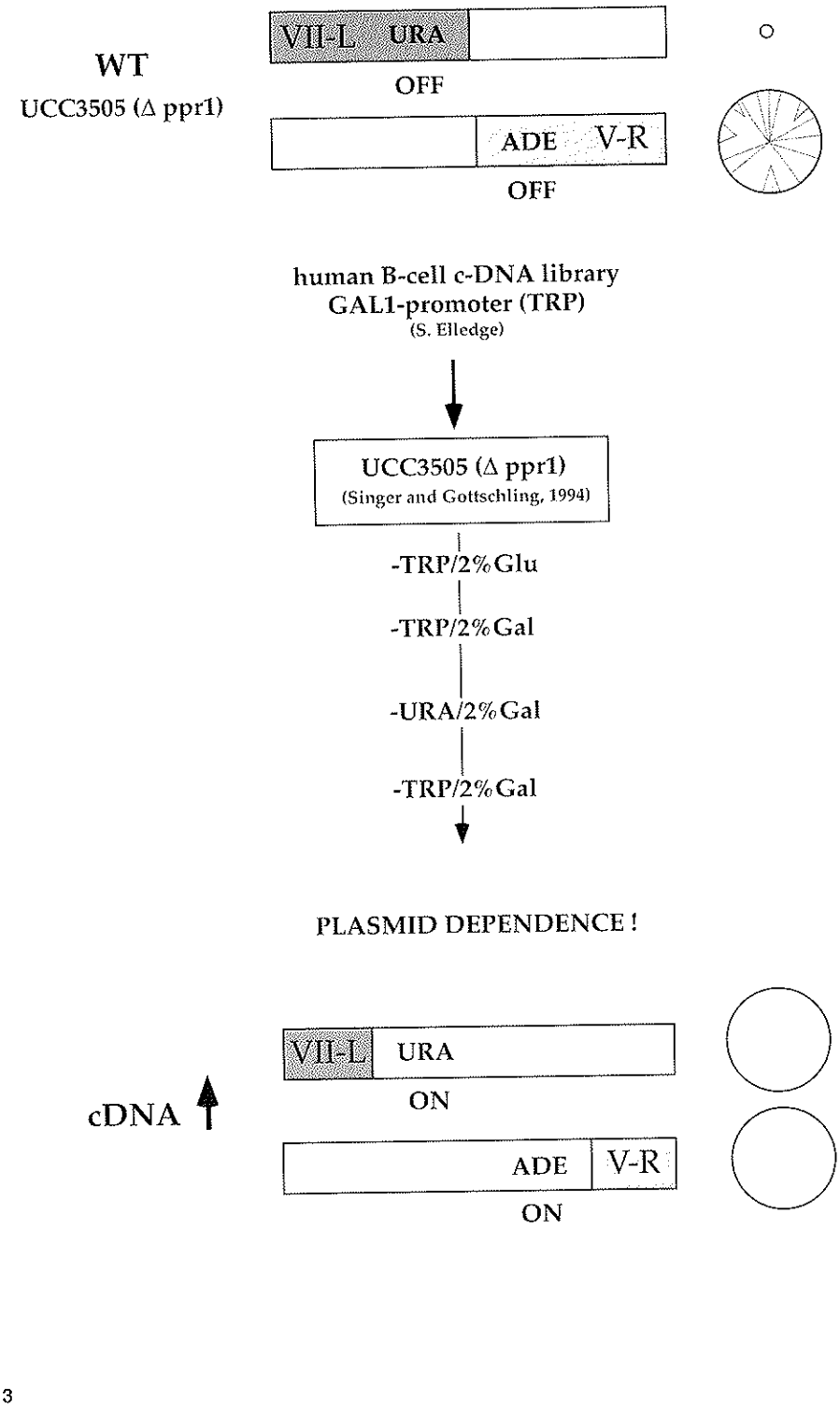

Figure 3

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Genetic principles of tumor suppression

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Introduction

Genome sites of putative tumor preventing genes have often been identified using genetic and physical mapping approaches, subsequent loss-of-heterozygosity (l.o.h.) analyses and positional cloning experiments. When applying such an approach to human neuroblastoma, it has previously become evident that the distal part of the human chromosome 1p arm harbors at least one gene that upon its loss confers susceptibility to this type of cancer. We and other groups were able to localize the critical region that may contain this gene to the chromosome 1p36 band. Subsequent work in our group has aimed at the fine mapping of a neuroblastoma consensus deletion, at the generation of recombinant DNA clones specific for this deletion in an appreciably high density and at the identification of putative candidate genes from this region.

When performing l.o.h. analyses in numerous primary neuroblastoma tumors, it became more and more obvious that allelic deletions in the tumors are either very large in size, i.e. covering at least substantial parts of the 1p36 region (approximately 30 Mbp in size) or may in other cases be so small that the limited set of available polymorphic DNA probes was incapable of detecting them. Consequently, we changed our experimental strategy to detect regionally more confined deletions as well as other, more subtle aberrations, such as inversions or translocations. From our previous microcloning experiments in 1p36-p35 we could fall back on a large number of region-specific microclones. These clones were generally not of use for l.o.h., since they mostly did not detect any polymorphic sites. However, they proved valuable in screening libraries of cosmids, P1 phage clones, yeast artificial chromosomes (YACs), and - more recently - PAC (P1 derived artificial chromosomes) clones.

Our large insert library screening yielded a large set of long genomic DNA clones covering a substantial part of the 1p36 region. In order to obtain detailed information on the localization of these clones, we performed an extensive mapping approach that involved several experimental strategies. Pulsed field gel electrophoresis (PFG) provided the opportunity to identify neighboring microclone markers upon their hybridization pattern on "fingerprint blots" (Barnas *et al.*, 1995). Furthermore, the long DNA clones were subjected to fluorescent in-situ hybridization (FISH) on normal and - most importantly - on aberrant chromosomes of tumor cell lines. The breakpoints of the 1p36 located aberrations represented valuable borders of small intervals or "bins" that provided important tools for fine mapping of the long clones. Also, an extensive number of known genetic markers and 16 known genes were included in the analysis. This comprehensive "Integral mapping" approach (see last year's report) resulted in a very detailed physical map that - given the constraints of whole genome mapping attempts or the lack of specific long clone collections in other groups - was much more precise than any other pre-existing map of this region. Our subsequent work has proven by now that this integral map is vital to our further approach towards a candidate gene in 1p36.

Searching for candidate genes in the neuroblastoma consensus deletion

Patrick Onyango, Karin Paiha and Elke Kleiner, in collaboration with Robert Kurzbauer

We have previously been able to determine a refined region of consistent allelic deletions in neuroblastomas by means of analyzing two very small interstitial deletions (see last year's report). One of the deletions was found in a neuroblastoma cell line, SK-N-AS; whereas the other resides in the constitutional genotype of a neuroblastoma patient, CH91-074 (material kindly provided by J. Biegel, Philadelphia, PA). Using 1p36 located YAC and P1 phage clones for FISH mapping, we showed that the dele-

tions found in these two independent sources were extensively overlapping, leaving a smallest region of overlap that may be as small as 2-3 Mb.

In the course of last year's work we aimed i) to localize this consensus deletion as precisely as possible using both known genetic markers as well as employing a set of new clones, ii) to saturate this region with long cloned DNAs, such as YACs, cosmids, P1 clones, and PACs, iii) to use the long clone DNAs for the identification, cloning and characterization of putative candidate genes of this region.

Using FISH we could map the borders of the consensus deletion relative to a series of known chromosomal markers: the centromeric side of the deletion is bordered by the gene loci CD30 and NPPA, the HTR1D gene and the anonymous locus D1S97 flank it on the telomeric side.

Importantly, when analyzing the neuroblastoma consensus deletion for the presence of any of the putative cancer associated genes postulated to date, we had to exclude all of them from the critical region: the TNF receptor type II gene (TNFR2), a retinoblastoma binding protein (RIZ); a tumor suppressor gene of the mouse with a homologue in 1p36 (DAN), and the dominant negative HLH gene ID3 (heir1, isolated in this laboratory) clearly map proximal. One candidate locus was mapped distal of the deletion: the CDC2L1 (PITSLRE) gene family cluster of CDC like kinases is clearly telomeric of the critical region.

The region of the consensus deletion includes a series of 40 microclone markers and/or D1S loci and three known genes, i.e. the non-neuron specific enolase (ENO1), carbonic anhydrase VI (CA6) and phosphoglycerate dehydrogenase (PGD). In addition, we mapped one of the expressed sequence tags (EST's) of the EST database, designated D1S274E, to this region.

As none of the known genes in the critical region could be functionally associated with the neuroblastoma phenotype, we set out to identify and characterize new genes from the consensus deletion. Given the size of approximately 2-3 Mb of consistently deleted DNA we estimated that the analysis of the entire chromosomal segment might involve the identification of 20 to 30 new genes. In order to reduce this workload and the number of candidate genes, we attempted to identify even more informative chromosomal aberrations in neuroblastoma cells than the two small deletions of SK-N-AS and CH91-074. As shown in the paragraphs below, we succeeded in localizing two balanced translocation breakpoints of neuroectodermal tumors to the consensus deletion. Both breakpoints seemed to be in close vicinity to each other. At this point we initiated two approaches towards isolation of the relevant gene: while a positional cloning approach was taken to clone the breakpoints on the genomic level, we independently selected P1 and YAC clones from the vicinity of the translocation breakpoints and identified gene elements encoded by them. The experimental approaches taken were: CpG island detection, identification of evolutionarily conserved DNA probes, cDNA pool hybridization/selection, and exon trapping.

The exon trapping experiments utilizing several different P1 phage clones led to the identification and cloning of two novel genes in the neuroblastoma consensus deletion to date: "ext1" (see **Fig. 1**), was isolated from a P1 clone distal of both breakpoints. Subsequent cDNA library screening with the exon trapped fragment led to the isolation of a partial cDNA of 3.8 kb (**Tab. 1**). When this clone was sequenced, it was discovered that the gene is highly homologous to a phosphatase gene family. In comparison, the second gene found, designated "ext2" (**Tab. 1**) does not share any homologies to known genes in the parts identified so far. ext2 was isolated as an exon fragment from a proximal P1 clone (**Fig. 1**); subsequently an apparently full length cDNA clone of 4.5 kb could be isolated for this gene (see **Tab. 1**).

A third gene, designated "chs1" (**Fig. 1**), was identified by cDNA library pool hybridization/selection using the P1 clone L1945: this clone revealed a 1.75 kb message on northern blots; a full length clone was subsequently isolated by screening a fetal brain cDNA library. Sequencing revealed a very high homology to the ENO1 gene, and intriguingly the gene was found to map to the same P1 clone (see **Figure 1**). However, the homology was discontinuous, suggesting either a differential splice product or a second, highly homologous gene locus. Furthermore, we identified substantial homology to sequence motifs of a hyman c-*myc* promoter binding protein. Studies to investigate the nature and identity of chs1 are in progress.

The search for evolutionary conserved DNA sequence fragments from the 1p36 specific microclone library has previously led to the identification of several clones with DNA sequences that are highly homologous at least in all mammalian genomes tested. We could map one of these clones, lambda1-VI/475, by PFGE linkage analysis to the neuroblastoma consensus deletion. cDNA library screening with this clone as a probe led to the isolation of a homologous cDNA that detects a roughly 8 kb message on northern blots (see **Table 1**). Two independent cDNA clones were subsequently isolated which are 6 kb and 5.3 kb in size, respectively; they overlap and may thus represent the entire transcript of the gene. DNA sequence analysis of the cDNA is in progress to determine any homology to known genes or to identify any functional motifs within this gene.

Subsequent experimental steps after having identified these partly novel genes will involve the analysis of sequence integrity in tumor genomes and a functional analysis by ectopic expression in tumor cell lines. Work is in progress to transform a series of neuroblastoma tumor cell lines with appropriate expression plasmids.

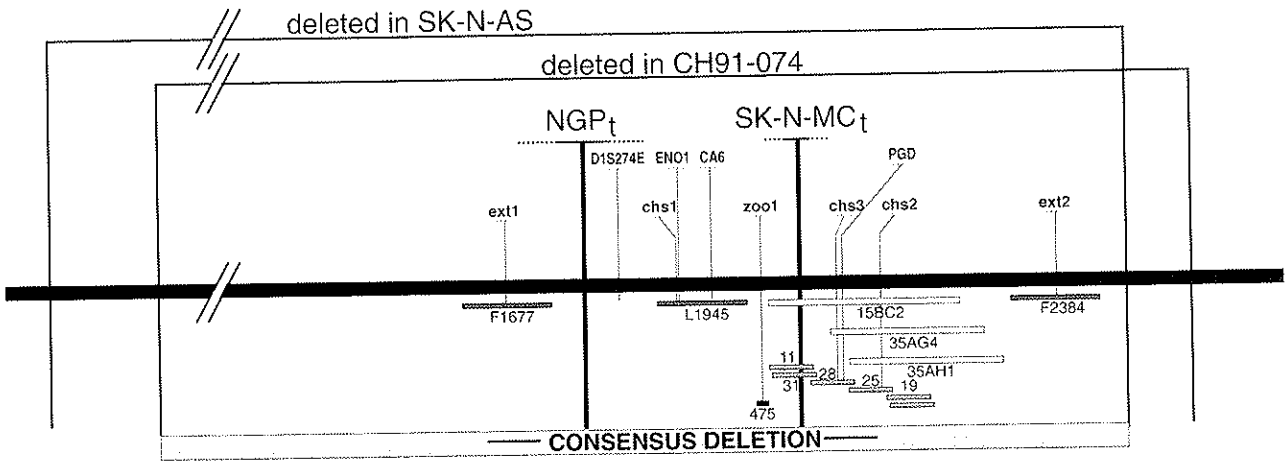

Fig. 1: Schematic representation of the 1p36.3 located consensus deletion in neuroblastomas. Important clones of this region are displayed as differently shaded horizontal bars: YACs in light grey, cosmids in middle and P1 clones in dark grey. The breakpoints of two balanced translocations, NGP and SK-N-MC, are displayed as prominent vertical bars. The other vertical bars represent the putative position of known and newly identified genes in the region.

Mapping, cloning and characterization of the translocation breakpoint in a neuro-ectodermal tumor cell line

Barbora Lubyova and Andreas Weith, in collaboration with
Robert Kurzbauer (I.M.P.) and Peter Ambros (CCRI Vienna)

In the course of mapping cytogenetic rearrangements such as deletion and translocation breakpoints to chromosome 1p36, we succeeded last year in localizing the breakpoint of a balanced translocation in a primitive neuroectodermal tumor (PNET) cell line, SK-N-MC. This tumor cell line had been diagnosed as neuroblastoma for more than 20 years, until the Ewing sarcoma specifc t(11,22)(q24;q12) was discovered in the karyotype of this tumor. Hence this tumor must be regarded a PNET. However, despite the common origin of both neuroblastoma and PNETs from neural crest it is important to note that many of the histochemical and pathological features of this tumor correspond to a neuroblastoma. Most importantly, the genome of these cells displays merely two further rearrangements in addition to the t(11;22), i.e. a trisomy for chromosome 8 and a balanced translocation t(1;X)(p36.3;p12). In order to rule out that the 1p36 translocation was an artifact acquired during *in vitro* cell culture, we had the chance to identify this aberration already in very early passage cells (kindly provided by J.Biedler, Sloan Kettering Memorial Inst., New York). This underlines the significance of the chromosome 1p36 specific rearrangement in this tumor cell line.

Our initial mapping attempts have pinpointed the SK-N-MC translocation breakpoint directly to the neuroblastoma consensus deletion at 1p36.3 (see **Figure 1**, see also last year's report). Further FISH analyses employing the set of YAC and P1 clones of the consensus deletion led to the identification of a three-YAC contig in the vicinity of the breakpoint (**Fig. 1**). Indeed, one of these YACs displayed a triple signal in the tumor cell line (**Fig. 2**), indicating that it is located across the rearranged DNA segment. In order to precisely localize the breakpoint and to obtain cloned fragments specific for it, we subcloned the respective YAC into cosmids and ordered the resulting clones again in a contig of overlapping fragments (see clones 11, 31, 28, 25, and 19 in **Fig. 1**). FISH analysis of these clones clearly showed that the two most telomeric clones of the contig displayed a triple signal on the SK-N-MC chromosomes. These two cosmids, 11 and 31, were almost entirely overlapping each other; it was, therefore, obvious that the translocation breakpoint of the tumor chromosome maps to the region common to these two cosmids.

With the aim to identify the candidate genes that may be affected by the rearrangement, those cosmid DNA fragments that were located in the immediate vicinity of the translocation breakpoint (31, 28, and 25) were used for gene identification strategies as already described above. When the cosmid DNA fragments were subjected to exon trapping experiments, no exon fragment could be recovered from any of the three clones employed. Also, digestion of the fragments with CpG island specific rare cutter restriction enzymes did not reveal any obvious cluster of restriction sites, hence CpG islands were probably not contained. However, when the cosmid DNA was used for the hybridization/selection of positive clones from PCR amplified cDNA libraries, three different transcribed elements were recovered (see

Table 1: Newly isolated genes from the neuroblastoma consensus deletion

name	isolation method	cDNA length	transcript size	homology
ext1	exon trapping	3.8 kb	6.0/4.5* kb	phosphatase
chs1	cDNA hybr./sel.	1.75 kb	1.75 kb	ENO1, c-myc promoter binding
zoo1	conserv.	6 kb + 5.3 kb	~ 8 kb	not determined
chs3	cDNA hybr./sel.	~ 0.3 kb	4.4 kb	none
chs4	cDNA hybr./sel.	~ 0.4 kb	2.4	identical with PGD
chs2	cDNA hybr./sel.	~ 0.3 kb	6.5 kb	none
ext2	exon trapping	4.5 kb	4.5 kb	none

loci are ordered telomere-centromere
* putative alternative splice

Table 1). All three fragments detected transcripts on northern blots when used as hybridization probes. When the identified cDNA fragments were subjected to DNA sequence analysis, it was revealed that one of the clones, mapping to the cosmid 28, corresponded to the phosphoglycerate dehydrogenase (PGD) gene. This was the only clone showing an unequivocal identity as a known gene. The second cDNA fragment, "chs2" could be localized centromeric to the PGD gene, mapping to cosmid 25! Northern blot hybridization with this clone clearly showed that this chs2 is not part of the PGD gene (compare the transcript sizes in **Table 1**). DNA sequence comparison revealed that this segment does not display any homology to any known gene sequence. We, therefore, conclude that the cDNA isolated corresponds to another new, yet unidentified gene.

A third gene could be identified on the same cosmid as PGD. This gene again shows a transcript that is different from PGD and thus may represent a different entity (**Tab. 1**). Homology searches in the DNA sequence databases merely outlined a homology of a part of the transcribed region to the characteristic sub-telomeric repeat elements. No additional conserved sequences indicating the possible function of this new gene were discovered. With respect to its posssible involvement in the chromsosomal rearrangement of SK-N-MC, a possible extension of this gene into cosmid 31 and thus into the region containing the translocation breakpoint is of major interest. At this point, the cDNA obtained from the cDNA hybridization/selection experiment represented a partial cDNA of the gene encoded; hence this could not be answered at this point. Screening experiments to isolate full length cDNAs specific for this gene from a fetal brain cDNA library are in progress.

No evidence for the presence of a genetic element has yet been obtained for cosmid 31, the cosmid that spans the translocation breakpoint directly. The DNA fragment appears to have no CpG island sequence and no exon that might be trapped. As a possible explanation for our failure to isolate a cDNA using the hybridization/selection technique we may have selected an inappropriate cDNA library as a source, or the abundance of any gene encoded by this clone may be insufficient.

Our subsequent approach aimed at the determination of potential open reading frames in the genomic cosmid 31 DNA as a parameter for the presence of any candidate gene. We thus intitiated a long range sequencing project of the entire cosmid fragment. The cosmid clone was shotgun subcloned into approximately 800 bp plasmid subclones, which were then sequenced from either side. Sequence assembly has led to a continuous DNA sequence of approx. 28 kb to date. This sequence will be used to apply gene finding software analysis, such as "gene finder", "GRAIL", or "BLAST".

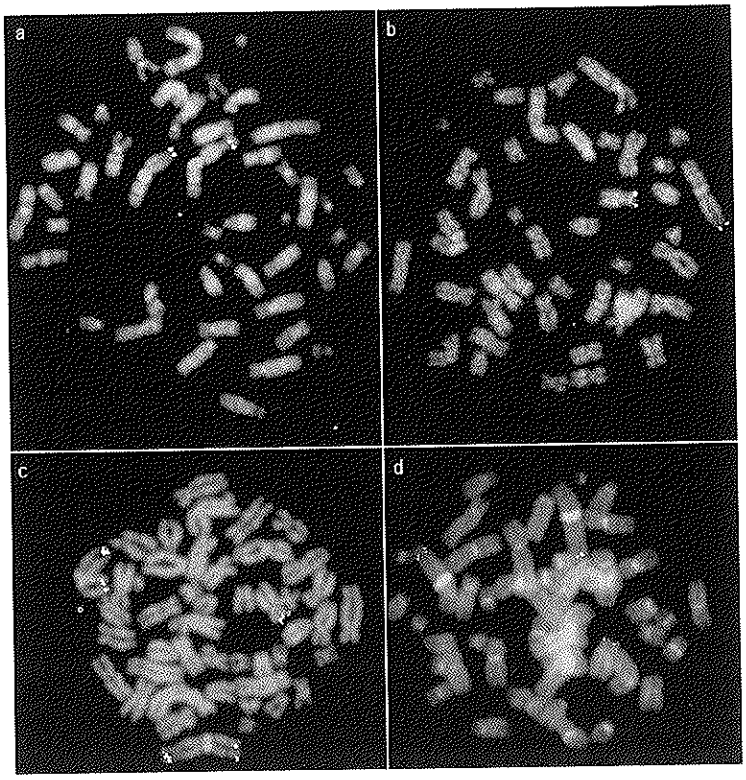

Fig. 2: FISH analysis of the balanced translocation in the PNET cell line SK-N-MC.
a: FISH signals (light spots) obtained by hybridization with a P1 clone probe that is specific for a locus proximal of the translocation breakpoint. Both chromosomes 1, wildtype and derivative, display hybridization signals.
b: FISH signals obtained with a P1 clone probe specific for a locus distal to the translocation breakpoint. The normal chromosome 1 and the derivative X chromosome are labeled.
c: Hybridisation signal obtained with a YAC clone that bridges the translocation breakpoint. FISH signals of the YAC probe are seen on both derivative chromosomes. Additional signals are visible on 1qdist due to chimerism of the YAC insert.
d: Cosmid subclone 31 bridges the translocation breakpoint: FISH with this clone as a probe generates a 1p hybridization signal pattern corresponding to that obtained by the YAC probe.

Fine mapping, identification, and molecular cloning of a translocation breakpoint in a neuroblastoma cell line

Paola Gardellin, Judith-A. Lummerstorfer, Elke Kleiner, Karin Paiha and Andreas Weith

The neuroblastoma cell line NGP has previously been described as having a rearrangement in the distal chromosome 1p36 region that corresponds to a balanced translocation. Indeed, a reciprocal exchange between chromosome 1 and 15 material has taken place in this cell line; however, a concomittant duplication of a yet unknown proportion of chromosomal material immediately proximal of the translocation breakpoint has led to a partial trisomy of chromosome 1p36 material. Our recent efforts to precisely localize the translocation and likewise the duplication breakpoint has revealed that the translocation breakpoint indeed maps to the region commonly deleted in the SK-N-AS cell line and the CH91-074 constitutional deletion (see above). This clearly points out the importance of an analysis of this breakpoint, as it is highly likely that the gene of interest is affected by this rearrangement. In contrast, during the FISH analysis it became apparent that the end point of the duplicated region is centromeric to the consensus deletion. This point, therefore, seems less likely to have affected a significant tumor-associated gene; we thus did not concentrate on the localization of that particular breakpoint.

FISH fine mapping using P1 and YAC clones of the consensus deletion indicated that the two translocation breakpoints of NGP and SK-N-MC were located close together within the consensus deletion (see **Figure 1**); however, due to the limited resolution of metaphase FISH a precise indication of the distance between the two probes is impossible to date. It became obvious that at least one P1 clone could be placed in the interval between the two rearrangements, as it mapped distal of the breakpoint in SK-N-MC and proximal to the NGP translocation breakpoint (**Fig. 1**). This shows that the minimum distance between the two translocations corresponds to the length of the P1 clone, i.e. 78 kbp. However,

more precise results await further investigations with PFGE mapping and additional FISH experimental approaches (see below).

The approach to precisely map the NGP translocation breakpoint itself was difficult owing to the duplicated region proximal of the breakpoint. Each probe residing in this region produced a FISH signal pattern that was compatible with the respective clone bridging the breakpoint. This forced us to try and approach the breakpoint from the telomeric region where clones exclusively yield a translocated hybridization pattern.

Major attempts are currently made to identify those clones that are closest to the translocation breakpoint. From our comparison of the two translocation breakpoints we argued that the P1 clone located between the two breaks was the closest available on the proximal side. A series of candidate loci was generally available for the distal side. The most valuable information for the closest proximal/distal clone pair was obtained from a recently developed technique, designated DIRVISH (direct visualization of in situ hybridization products). This procedure makes use of extended native chromatin stretched out of interphase nuclei as a target for fluorescent in situ hybridization. Any probe derived from longer clones such as cosmids, P1 clones or YACs will generate an extended hybridization signal on this chromatin. This provides the possibility to precisely map adjacent or even overlapping DNA clones. The length of the fluorescent signals correspond to the type of clones, i.e. comid clones generate shorter signal stretches than P1 clones or even YACs. Furthermore, the technique appears to allow the establishment of a relative order of adjacent probes and even the determination of approximate distances in the kilobasepair range and degrees of overlaps.

DIRVISH enabled us to generate a very detailed map including the long DNA clones around the NGP translocation breakpoint. Hybridization with two differently labeled, adjacent probes readily revealed that the two closest probes either side of the breakpoint leave only a very small gap in between them. We are thus in the process of screening a chromosome 1-specific cosmid library (obtained from the Reference Library Database in Berlin) with suitable DNA probes. The analysis of positive clones obtained in a first screening experiment is in progress.

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Molecular cell biology of hematopoietic cells

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Introduction

The generation of hematopoietic cells involves the highly ordered and controlled proliferation of immature progenitor cells and their commitment and differentiation into fully mature cells of various lineages. This process is tightly controlled to ensure that all cell types of the hematopoietic system are produced in a balanced fashion according to the needs of the organism. In leukemias, for example, such normal control mechanisms are bypassed, leading to a massive and uncontrolled outgrowth of largely immature hematopoietic cells that cause the disease.

Our group studies molecular mechanisms of hematopoietic cell differentiation and how such mechanisms are bypassed in the pathogenic state. These studies are mainly performed in a well-established experimental model system using oncogene transformed bone marrow cells of chicken. In this system, retroviral oncogenes are employed to transform early hematopoietic progenitors from chicken bone marrow which can be propagated *in vitro* under well-defined culture conditions; inactivation of oncogene activity induces their differentiation. Normal untransformed chicken and human hematopoietic progenitor cells grown in the presence of cytokines are also used.

In 1995 we continued our efforts to study gene expression in erythroid cells and antigen-presenting dendritic cells. Additionally, various gene transfer techniques were tested for efficient transfer and gene expression in dendritic cells to develop gene-modified antigen-presenting dendritic cells.

1. MOLECULAR BIOLOGY OF RED BLOOD CELL DIFFERENTIATION

P. Bartunek, N. Koritschoner and G. Stengl

In 1995, we extended our studies on the activity of the c-erbA/T3R, RAR and RXR receptors and of the GATA transcription factors in red blood cells. The regulation of c-myb proto-oncogene expression during red blood cell differentiation was also investigated. The c-erbA/T3R, RAR and RXR serve as ligand-inducible transcriptional regulators of erythroid-specific gene expression, and following addition of hormones accelerate red blood cell differentiation. The GATA proteins belong to a class of transcription factors that bind to a large number of erythroid-specific genes and regulate their activity. In previous studies we found that GATA-1 accelerates terminal red cell differentiation, while GATA-2 has an important function in red cell progenitors, thus demonstrating differential functions of GATA-1 and GATA-2 in red blood cell development (Briegel *et al.*, 1993; 1995; in collaboration with H. Beug, IMP, Vienna, Austria and D. J. Engel, Northwestern University, Evanston, Chicago, USA).

Last year, we reported the identification of 3 major GATA-1 binding sites in the c-myb promoter that might be involved in repression of c-myb expression. We have now analyzed changes in chromatin structure occurring in the c-myb locus in the course of red cell differentiation of SCF (stem cell factor)-dependent erythroid progenitors. This study led to the identification of a DNAseI hypersensitive site that was dramatically induced during differentiation with kinetics correlating with the downregulation of c-myb. We thus speculate that this region might be directly involved in silencing c-myb expression which is a

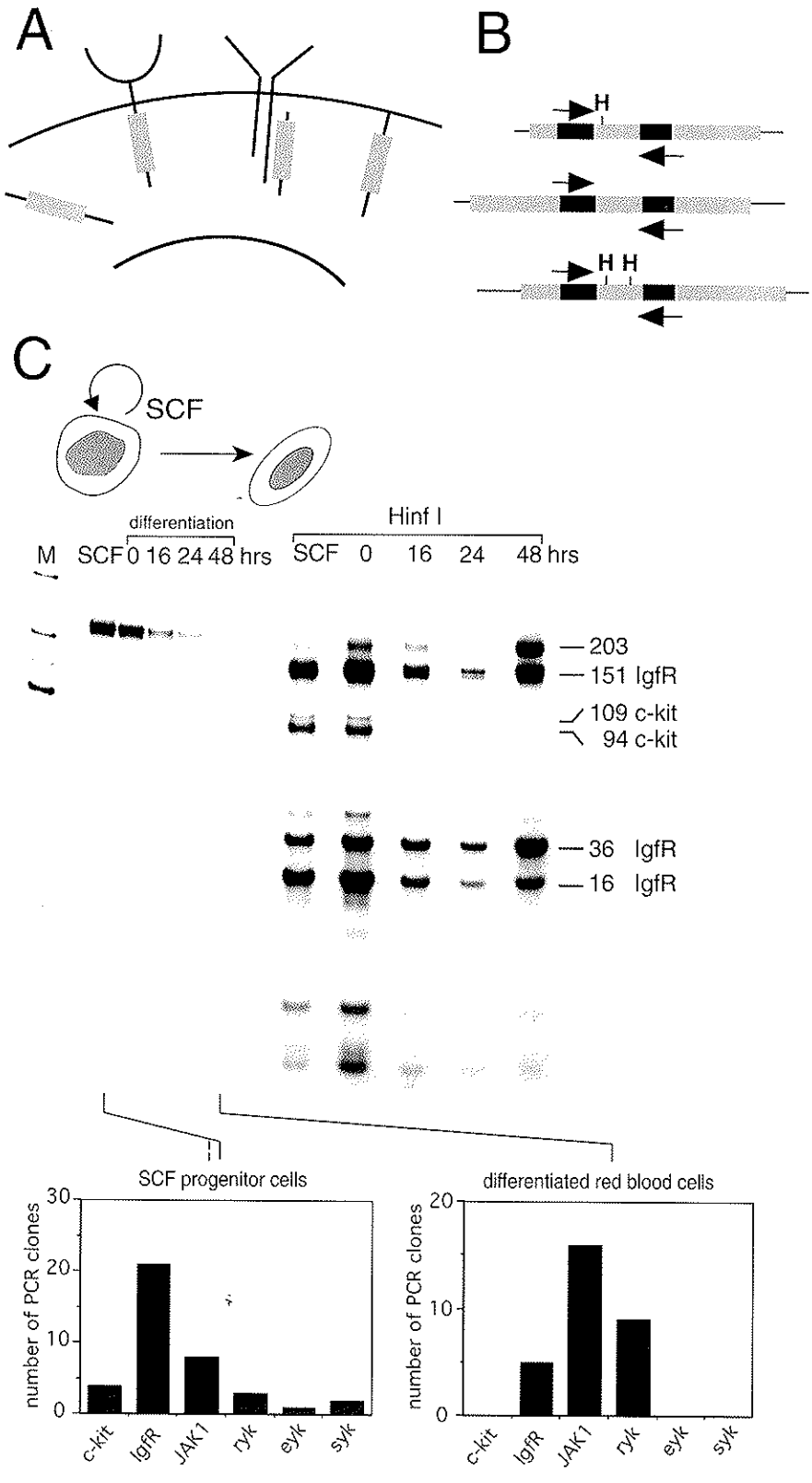

Fig.1: Analysis of red cell-specific gene expression by domain-directed differential display.
(A) Receptor and non-receptor tyrosine kinases are schematically shown. The conserved kinase domain is represented by a box. (B) PCR amplification of members of the tyrosine kinase family. The primers used are indicated (arrows). Restriction endonuclease cleavage sites (H). (C) SCF-dependent erythroid progenitor cells were induced to differentiate for various periods of time as indicated and subjected to domain-directed differential display. The PCR products prior to and after digestion with the restriction endonuclease Hinf I were terminally labeled by Klenow enzyme and ^{32}P and analyzed by gel electrophoresis. Additionally, PCR products of undifferentiated and differentiated (48 hrs) SCF progenitors were subcloned in plasmid vectors and subjected to sequence analysis.

prerequisite for normal terminal differentiation. This region is now more extensively analyzed using different reporter gene constructs and studying DNA-protein interactions.

As a new approach to analyze red cell-specific gene expression we employed domain-directed differential display (**Fig. 1**). This technique enables us to determine the expression pattern of multigene families (e.g. of tyrosine kinases) in undifferentiated and differentiated red cells. Therefore, RNA of undifferentiated chicken SCF-dependent erythroid progenitor cells and of cells after various periods of differentiation is subjected to reverse transcription using random hexamers; cDNAs are amplified by PCR using a set of tyrosine kinase-specific primers. To reveal individual members of the tyrosine kinase family, the PCR products are digested with various restriction endonucleases and the DNA fragments obtained are displayed on gels.

Fig. 1 shows that chicken red cell progenitors and mature red cells exhibit a distinct pattern of bands which is indicative of differential expression of members of the tyrosine kinase family in undifferentiated and differentiated cells. The identity of the tyrosine kinases involved is readily determined by DNA sequencing of the PCR products. We have now applied this approach also to analyze the expression profile of other multigene families in these cells. In the course of our studies we expect to obtain already known genes but also to discover new members of these multigene families that might represent important regulators of red cell differentiation.

2. ANTIGEN-PRESENTING DENDRITIC CELLS

2.1. Cell biology of antigen-presenting dendritic cells.

J. Madruga and S. Diebold

Dendritic cells are found at various locations within the organism, particularly in those areas which are potential entry sites for pathogens (like e.g. the skin where they are called Langerhans cells). They represent a cell population whose function it is to capture antigens, migrate to the lymphoid organs and present the processed antigens to T lymphoid cells. In previous studies we developed a powerful *in vitro* differentiation system for antigen-presenting dendritic cells based on the v-rel estrogen receptor (ER) fusion protein v-relER (Boehmelt *et al.*, 1992; 1995; in collaboration with P. Enrietto, Stony Brook, New York, USA).

v-rel, the transforming oncogene of the avian **r**eticulo**e**ndotheliosis **v**irus (REV-T), is the oncogenic version of c-rel and belongs to the NF-κB/rel/dorsal family of transcription factors. The conditional, hormone-inducible v-relER causes v-rel-specific but otherwise unaltered transformation of chicken bone marrow cells *in vitro*. Such v-relER transformed cells can be grown as clonal and homogenous cell populations in the presence of estrogen when the v-relER oncoprotein is active. "Switching off" v-relER activity by administration of an estrogen antagonist allows their differentiation. Depending on the specific culture conditions used, cells will differentiate into fully competent antigen-presenting dendritic cells or into cells resembling mammalian neutrophils (Boehmelt *et al.*, 1995).

Interestingly, v-rel and undifferentiated v-relER cells exhibit some properties of lymphoid cells. They express the B cell-specific activator protein BSAP (in collaboration with Petra Dörfler and Meinrad Busslinger, IMP, Vienna), contain one rearranged immunoglobulin light chain allele and exhibit surface immunoglobulin M (IgM). During this year, we investigated the B-lymphoid phenotype of v-relER cells in more detail. It was found that v-relER cells, both undifferentiated and differentiated, do not produce immunoglobulins by RNA and protein analysis. Rather, they present serum-borne IgM apparently via Fc receptors on their cell surface that is detected by FACScan analysis. IgA is also detected. However, it remains puzzling that v-relER cells do contain a rearranged light chain gene, since this points towards a lymphoid origin of these cells. However, their lymphoid properties are rather weak. For instance, BSAP expression in v-relER cells is approximately 50 times lower than the activity detected in chicken bursa and down-modulated or lost when v-relER cells differentiate.

We now propose a model which is compatible with our observations. In such a model (**Fig. 2**) a cell at an early stage of lymphoid development switches to a dendritic cell progenitor state upon transformation with v-relER. Inactivation of v-relER allows this progenitor to diffentiate into dendritic cells or granulocytes. A common progenitor for macrophages, dendritic cells and granulocytes and a bipotential precursor for B cells and macrophages have been found in murine hematopoietic tissues. Furthermore, a lineage switch from B-lymphoid cell lines to macrophages was reported by Klinken *et al.*, 1988. Lindeman *et al.*, 1994, described another example of genetic reprogramming from B cells to neutrophilic granulocytes in a transgenic mouse strain. Therefore, all these observations point towards a close relationship between the myeloid and B-lymphoid cell lineages and strongly support the notion that the hematopoietic system is more flexible than initially thought.

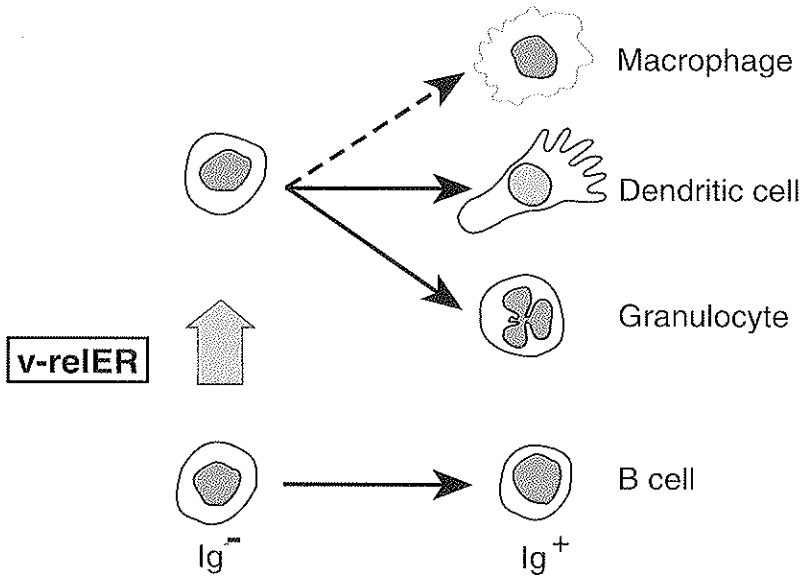

Fig 2.: Model of v-relER-induced lineage switch: A B-lymphoid cell with one immunoglobulin light chain allele rearranged (Ig-) switches to a myeloid cell differentiation program by transformation with v-relER. This cell now acquires the potential to differentiate into dendritic cells or neutrophilic granulocytes. Its capacity to differentiate into macrophages still remains to be determined.

2.2. Molecular biology of antigen-presenting dendritic cells.

N. Koritschoner, J. Madruga, P. Bartunek and S. Knespel

Our system for differentiation of v-relER cells into dendritic cells *in vitro* is particularly appealing, since it allows for the first time an analysis of the gene expression repertoire required for the dendritic cell differentiation program. The availability of clonal cell populations that can be expanded to large cell numbers and induced to differentiate into dendritic cells at will is an important advantage of the system, since otherwise dendritic cells are difficult to isolate and the populations obtained are not pure. Thus, dendritic cell specific genes can be readily identified by PCR-based differential cDNA cloning. We expect that our analysis will point out important regulator genes involved in differentiation and maturation of dendritic cells. Such studies are currently being performed by using cloning strategies similar to the ones described for red cell progenitor differentiation (see above).

2.3. Gene transfer into antigen-presenting dendritic cells.

S. Diebold

While it is well established that dendritic cells represent highly active professional antigen-presenting cells that capture, process and present antigens, it is still a matter of debate how the different types of dendritic cells (Langerhans cells in skin, "veiled" cells, interdigitating cells, or follicular dendritic cells) relate to each other. In mice, dendritic cells can be obtained from bone marrow and spleen (Inaba *et al.*, 1992; 1993). In humans, dendritic cells are generated from $CD34^+$ peripheral blood stem cells treated with GM-CSF and TNFα (Caux *et al.*, 1992). Alternatively, a mononuclear cell fraction from peripheral blood is cultured in the presence of GM-CSF plus IL-4 (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994). To determine how the v-relER dendritic cells relate to these cells, human dendritic cells were isolated by either protocol and grown in the presence of the appropriate cytokines (in collaboration with R. Berger, AKH, Vienna, Austria and A. Pezzutto, MDC, Berin, Germany). Cells were extensively studied for cell surface marker expression, mobility and their activity in mixed lymphocyte reaction. Our studies indicate that these protocols yield two types of dendritic cells exhibiting a similar surface marker expression pattern but differ markedly in morphology and motility. Whether this represents different stages of maturation and/or different functional activities still remains to be shown. Finally, v-relER dendritic cells resemble more peripheral blood-derived dendritic cells, both by morphology and motility.

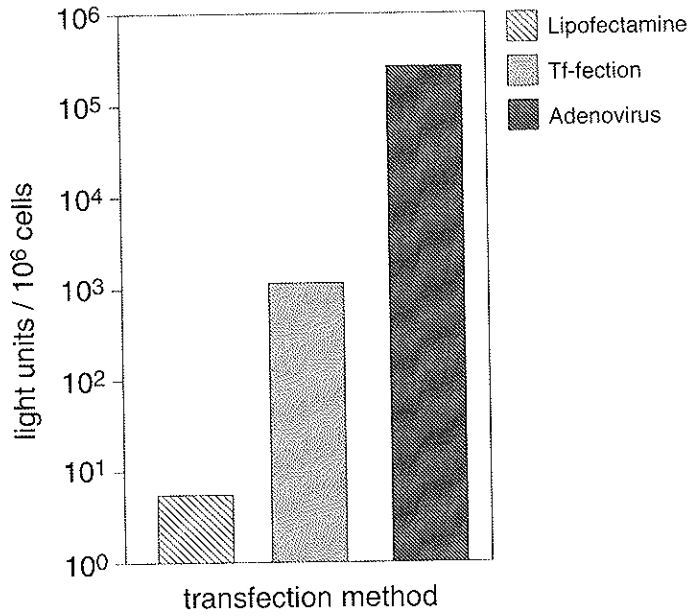

Fig.3: Gene transfer into human peripheral blood-derived dendritic cells. A luciferase gene under control of the CMV promoter was used for lipofection (Lipofectamine, GIBCO BRL) and transferrinfection. A replication-deficient adenoviral vector containing the luciferase gene under CMV promoter control was constructed by H. Lehrmann and M. Cotten, (IMP, Vienna, Austria). Luciferase expression was measured 48 h after transfection.

Additionally, our efforts are directed towards establishing an efficient gene transfer system for dendritic cells. Standard methods like lipofection (Lipofectamine, GIBCO BRL) yielded low expression levels of the luciferase reporter gene due to the high toxicity of the agent. Gene transfer and expression by transferrinfection was moderate (in collaboration with E. Wagner, Bender & Co, Vienna, Austria, and M. Cotten, IMP, Vienna, Austria). So far, the highest transfection efficiency was obtained with an adenovirus vector (**Fig. 3**; in collaboration with H. Lehrmann and M. Cotten, IMP, Vienna, Austria). These studies should eventually enable us to generate gene-modified dendritic cells that express e.g. tumor-specific epitopes to elicit a tumor-specific immune response and thus can be employed for immuno tumor therapy.

Publications during the year

Bartunek, P., Pichlikova, L., Stengl, G. Boehmelt, G. Beug, H., Dvorak, M. and Zenke, M. (1996). Avian stem cell factor (SCF): Production and characterization of the recombinant His-tagged SCF of chicken and its neutralizing antibody. *Cytokine* **8**, 14-20.

Beug, H., Bartunek, P., Steinlein, P. and Hayman, M. (1995). Avian hematopoietic cell culture. In vitro model systems to study oncogenic transformation of hematopoietic cells. *Methods in Enzymology* **254**, 41-76.

Boehmelt, G., Madruga, J., Dörfler, P., Briegel, K., Schwarz, H., Enrietto, P. and Zenke, M. (1995). Dendritic cell progenitor is transformed by a conditional v-rel estrogen receptor fusion protein v-relER. *Cell* **80**, 341-352.

Boehmelt, G., Stengl, G., Enrietto, P. J. and Zenke, M. (1995). v-rel protects transformed chicken bone marrow cells from apoptosis: A study with the conditional v-rel estrogen receptor fusion oncoprotein v-relER. In: Advances in Avian Immunology Research, Davison, T. F., Bumstead, N., Kaiser, P., eds., Carfax Publishing Comany, pp.65-73.

Briegel, K., Bartunek, P., Stengl, G., Lim, K.-C., Beug, H., Engel, J.D. and Zenke, M. (1995). Regulation and function of transcription factor GATA-1 during red blood cell differentiation. Submitted.

Dolznig, H., Bartunek, P., Nasmyth, K., Müllner, E.W. and Beug, H. (1995). Terminal differentiation of normal chicken erythroid progenitors: Shortening of G1 correlates with loss of D-cyclin/cdk4 expression and altered cell size control. *Cell Growth and Diff.* **6**, 1341-1352.

Other references

Boehmelt *et al.*, 1992, *EMBO J.* **11**, 4641-4652.

Briegel *et al.*, 1993, *Genes Dev.* **7**, 1097-1109.

Caux *et al.*, 1992, *Nature* **360**, 258-261.

Inaba *et al.*, 1992, *J. Exp. Med.* **176**, 1693-1702.

Inaba *et al.*, 1993, *Proc. Natl. Acad. Sci. USA* **90**, 3038-3042.

Klinken *et al.*, 1988, *Cell* **53**, 857-867.

Lindemann *et al.*, 1994, *Immunity* **1**, 517-527.

Romani *et al.*, 1994; *J. Exp. Med.* **180**, 83-93.

Sallusto and Lanzavecchia, 1994; *J. Exp. Med.* **179**, 1109-1118.

Animal house

Scientific coordinator	Erwin F. WAGNER
Veterinarian	Karl SCHELLANDER (Univ. Vienna)
Chief technician	Melanie KING
Technician	Mijo DEZIC
Technician	Susanne GRATZL
Technician	Ryzard KUPINSKI
Technician	Erika WEISSENGRUBER
Part-time assistant	Dominik MAYR

The animal house provides technical support for the different research programs of the I.M.P. At present, mice, rabbits, birds and *Xenopus* are housed within the facility.

The animal house has the capacity to house up to 12,000 mice that are either inbred, outbred, random bred or F1 hybrid. These also include congenic and mutant stocks that are bred and maintained for in-house use. A large variety of transgenic and knock-out stocks are also bred as part of the research projects. Additional mice for short-term projects are bought-in due to space limitations. We have our own breeding flock of 40 outbred chickens which produces fertile eggs for embryo work, live birds for other projects and youngstock to replenish the flock. Approximately 32 *Xenopus laevis* are maintained and used for research work, together with 20 bought-in rabbits for the production of antibodies.

A new rabbit room with a separate room for experimental procedures was completed and is now in full operation. The new room has facilitated the opportunity to improve the environmental enrichment of the rabbits.

The animal house staff perform a variety of technical procedures for the research projects. These include the administration of substances to the animals by various routes such as intra-venous, intra-peritoneal and sub-cutaneous. The collection of blood and other tissues from adults, immature and foetal animals is also offered. Surgical procedures such as tumor implantation, vasectomy and hysterectomy, together with non-surgical procedures such as tumor measuring are also carried out. All procedures are performed to a high standard under the appropriate anaesthetic regime and with the necessary licences. The animals are used by the following scientists:
Mouse studies: Max Birnstiel, Erwin Wagner, Denise Barlow, Gerhard Christofori, Thomas Jenuwein and Meinrad Busslinger.
Chicken studies: Hartmut Beug and Martin Zenke
Xenopus studies: Max Birnstiel.
Rabbit work: Hartmut Beug, Thomas Jenuwein.

Protein modeling

Scientist	Anton BEYER
PhD student	Aron MARCHLER-BAUER (on temporary leave until 31. 5. 95)
Diploma student	Wolfgang BITOMSKY (until 31. 3. 95)

Introduction

The main interest of the people working in this group is the relationship between amino acid sequence and tertiary fold of globular proteins.

Atomic coordinates from crystal structures can be used to construct a potential of mean force. Aron Marchler-Bauer has developed new methods for constructing such a potential. Besides data from known crystal structures he also uses information from sequence data alone. Applying this new matrix method for constructing the potential, the probability of finding the right structure for a given sequence is significantly increased. This work was part of a thesis which will be submitted in early 1996.

For storing and manipulating structural data of proteins we are using a relational database system. In this system the data are stored in a compact and consistent way and can be used for protein modeling projects and for statistical analysis. This database system is now running on a Silicon Graphics computer using ORACLE as database system. The number of structures currently available in the public domain is about 4000.

Wolfgang Bitomsky, a diploma student, performed molecular dynamics simulation studies to investigate structural differences and the flexibility of ras.p21.GDP and ras.p21.GTP. A more detailed description of this work can be found in the IMP yearbook 1994.

Fold recognition with efficient potentials of mean force

Aron Marchler-Bauer

One part of last years' work was aimed at increasing the efficiency by which statistically derived potentials of mean force can discriminate between correctly and incorrectly folded protein chains. To account for most of the factors stabilizing native protein structures, three types of potentials are calculated and combined:

(1) residue-pair interaction potentials

(2) potentials for the interaction with the solvent

(3) terms accounting for the local dihedral conformation of a residue.

The pair-potentials and solvent-terms are both calculated from the same statistics of Cβ-Cβ distances and can be combined without the need of relative scaling. Also, dihedral terms are treated in an analogous fashion and added to the total. Analysis of energies calculated with this set of potentials reveals that they complement each other quite well. To evaluate performance, the calculated potential sets are jack-knife tested in a simple non-gapped alignment (threading) experiment, where the aim is to pick out the native fold from a considerable amount of non-native folds. The percentage of correctly identified native folds as well as the average Z-score of the native structures during the search are used as quality criteria.

A method which utilises Mutation Data Matrices to smooth raw statistical data was shown earlier to improve the performance of the individual potentials (Bauer & Beyer, 1994). The effectiveness of this method decreases with the size of the database used. A treatment of redundancy in data-sets was devised, which is both simple and effective, allowing the use of many homologous structures without including any bias. As a consequence, the massive amount of information leads to more accurate potentials.

Two major applications of the potential sets were investigated: (I) Fold recognition and (II) Exploration of sequence space.

In (I) Fold recognition one tries to identify, whether a compatible fold for a protein of unknown structure can be found in the database of structural templates available. An alignment algorithm was developed, and tested, which uses dynamic programming and the 'frozen approximation' of residue environment, allowing for fast searching against the database. It was found that the occurrence of false positives in database searches remains the major problem of these approaches (Marchler-Bauer, 1995).

(II) To explore the 'sequence space' of a protein structure, an optimization scheme was devised, which changes random amino acid sequences to fit optimally on a given protein three-dimensional structure,

as predicted by the potentials. It was found that the optimized sequences barely resemble the native sequence and fall into the 'twilight zone' of statistical insignificant similarities, when compared with sequence-based methods. A large number of such optimised random sequences, however, can be used to compile sequence profiles, which turned out to be highly specific for members of the protein family to which the target three-dimensional structure belongs. Applications of such structure-derived and structure-specific sequence profiles in fold recognition and the characterization of protein families are investigated.

Publications during the year

Bitomsky, W. (1995). Molecular dynamics calculations of ras.p21.GDP and ras.p21.GTP. Diploma thesis, University of Vienna.

Habermann, B. (1995). Statistics and evaluation of correlation between residual temperature factor, variability of sequence, surface accessibility, and residual empirical energy of proteins. Diploma thesis, University of Vienna.

Marchler-Bauer, A. (1995). Protein Fold Recognition. *In: Proceedings of the International Conference on Molecular Structural Biology, Vienna, September 17-20, 1995.*

Marchler-Bauer, A., Salzer, U. and Prohaska, R. (1995). A universal family of membrane associated adaptor proteins. Submitted.

Martinez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. (1995). The Saccharomyces cerevisiae zinc finger proteins MSN2 and MSN4 are required for transcriptional induction through the stress response element (STRE). Submitted.

Other references

Bauer, A. and Beyer, A. (1994). An Improved Pair Potential to Recognize Native Protein Folds. *Proteins* **18**, 254-261.

Service department

Scientist	Gotthold SCHAFFNER
Technician	Elisabeth AIGNER
Technician	Ivan BOTTO
Technician	Robert KURZBAUER
Technician	Karl MECHTLER (since Oct. 95)
Technician media kitchen	Christa CZAPKA
Technician media kitchen	Gabriele BOTTO

Introduction

The Service Department offers a variety of rapid services to I.M.P. scientists. A large portion of our effort involves DNA sequencing, oligonucleotide synthesis and peptide synthesis. In addition, a modest amount of service is provided to institutions outside the I.M.P. We sequenced DNA probes, synthesized oligonucleotides and provided oligopeptides for university institutes in Vienna.

We also give help in designing immunization protocols, test titers of antisera and took over some of the duties in the new rabbit facilities in the Animal House. This service is possible only with the help of the Animal House staff and their animal breeding and maintenance facilities.

The Media Kitchen expanded into an additional room in 1995. The reorganization was necessary because of the increasing demand for diverse reagents and cell culture media. The quality and reliability of various solutions prepared by our Media Kitchen staff is vital for the continuous success of cell culture and other high standard work done in the Institute.

We also prepare many selected reagents like DNA molecular weight markers, enzymes, transformation-competent *E.coli* strains and keep stock of cloning vectors, primers etc.

Oligonucleotide synthesis

This year, we synthesized about 2500 oligonucleotides, 700 more than last year (**Fig. 1**). This substantial increase was mainly due to PCR primers and short oligonucleotides used for two large sequencing projects as is clearly visible in the graph (**Fig. 2**, e.g. January).

There was again a heavier demand just before holiday seasons. But, with the second oligonucleotide synthesizer (PERKIN-ELMER/ APPLIED BIOSYSTEMS 394) installed in late fall 1994, we had no problem to increase our synthesis capacity.

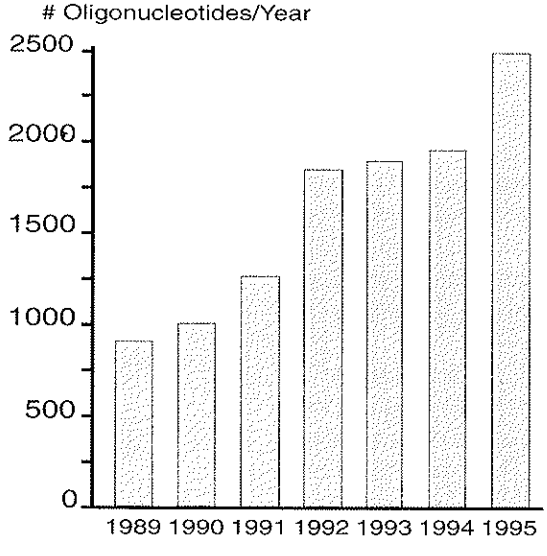

Fig. 1 : Total amount of oligonucleotides synthesized per year.

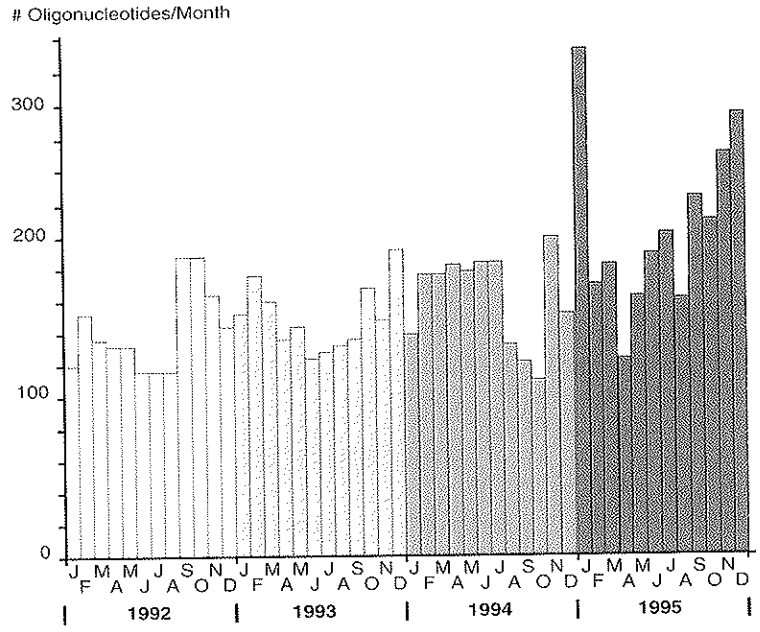

Fig. 2: Number of oligonucleotides synthesized per month of the period 1992-1995.

Peptide synthesis

Since October 1995 peptide synthesis has been a part of our services. Previously, oligopetides were provided by Ernst Wagner's group on a collaborative basis. Oligopeptide synthesis itself has become a more routine procedure in recent years, but the isolation of the pure peptide product is often very laborious. This is the main reason for the relatively small number of peptides synthesized so far.

Sequencing

Two sequencing projects, the genome of a 44 kbp chicken adenovirus (CELO) and 40 kbp fragment of human chromosome 1 with a very high alu-repeat content contributed to the 6800 + samples sequenced this year (**Fig. 3**). This represents a sequence length of about 3 Mbp!

Two different sequencing strategies were used: in the CELO project we sequenced exo III deletion clones, starting with EcoRI and HindIII subclones of the viral genome. The gaps were closed by "primer walking". The entire viral genome was sequenced revealing a number of unexpected details of this virus (see report of Cotten, and Chiocca *et al.*, 1996). The human cosmid clone was sequenced by starting with subclones made via a "shot gun" approach. The Chromosome 1 project is nearing completion. Comparison of the techniques used in these two projects should allow us to decide which one to employ in future efforts.

It became clear that with the chromosome 1 cosmid clone containing highly repetitive sequence motifs we needed a system that could read at least 500 nucleotides in a reaction. The new PERKIN-ELMER/APPLIED BIOSYSTEMS 377 DNA sequencer does this reproducibly. In **Fig. 4** we compared two sequencing reactions with the same DNA sample and primer analyzed on a 373A and 377. The resolu-

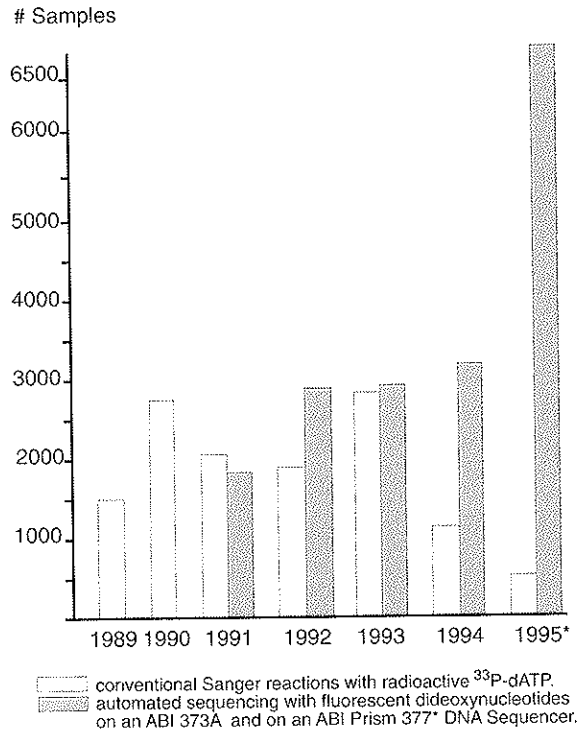

Fig. 3: Total numbers of samples sequenced in 1989 - 1995. The darker filled bar of 1995 (*) represents numbers of automated sequencing done on both ABI 373A and 377 DNA sequencers.

tion on a 377 is much better. We therefore upgraded the 373A in December this year in order to get comparable results. The dideoxy technique with fluorescently labeled dideoxynucleotides and new Taq polymerases combined with a thermostable pyrophosphatase has also become so reliable and straight forward that the number of conventionally sequenced samples dropped to about 500 this year (**Fig. 3**).

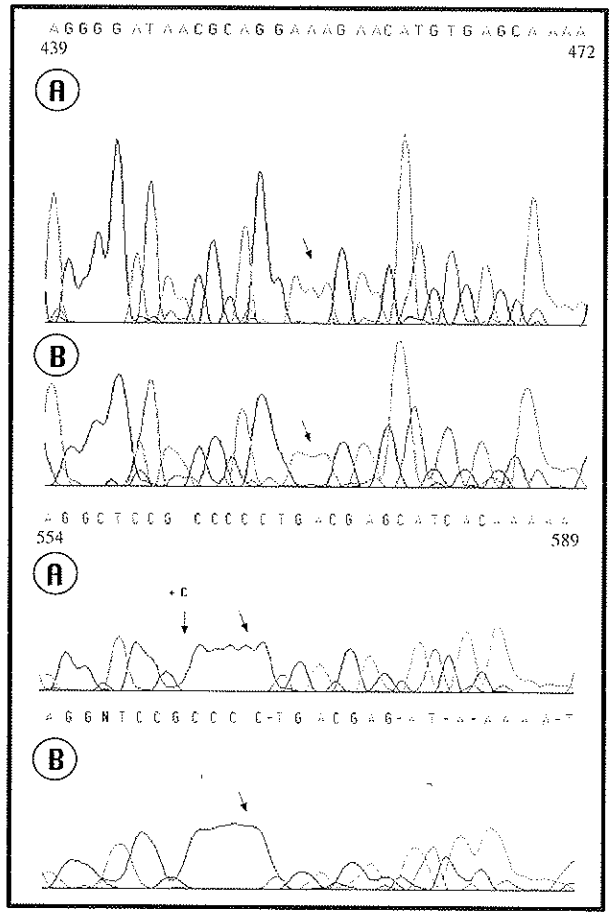

Fig. 4: Comparison of sequences analyzed on a ABI Prism 377 DNA sequencer (A) and ABI 373A DNA sequencer (B). Streches of A´s and C´s are marked with arrows. Differences are obvious in panel A vs. B in the lower panels (nucleotide # 554 to 585). In panel A a single C is missing, whereas 5 nucleotides are missing and one ambiguity is present in panel B.

Publications during the year

Buschle, M., Cotten, M., Kirlappos, H., Mechtler, K., Schaffner, G., Zauner, W., Birnstiel, M.L. and Wagner, E. (1995). Receptor-mediated gene transfer into human T lymphocytes via binding of DNA/CD3 antibody particles to the cd3 T cell Receptor complex. *Human Gene Therapy* **6**, 753 - 761.

Chiocca, S., Kurzbauer, R., Schaffner, G., Baker, A., Mautner, V. and Cotten, M. (1996). The complete DNA sequence and genomic organization of the avian adenovirus CELO. *J. Virol.*, in press.

Ellmeier, W., Barnas, Ch., Kobrna, A., Kleiner, E., Kurzbauer, R. and Weith, A. (1995). Cloning and characterization of CpG islands of the human chromosome 1p36 region. *Genomics* , in press.

Kain, R., Matsui, K., Exner, M., Binder, S., Schaffner, G., Sommer, E.M., and Kerjaschki, D. (1995). A novel class of antigens of anti-neutrophil cytoplasmic antibodies (ANCA) in necrotizing and crescentic glomerulonephritis: The lysosomal membrane glycoprotein h-lamp-2 in neutrophil granulocytes and a related membrane protein in glomerular endothelial cells. *J. Exp. Medicine* **181**, 585 - 597.

Mayr, B., Schaffner, G., Kurzbauer, R., Reifinger, M. and Schellander, K. (1995). Sequence of an exon of tumour suppressor p53 gene - a comparative study in domestic animals: mutation in a feline solid mammary carcinoma. *Br. vet. J.* **151**, 325 - 329.

Mayr, B., Schaffner, G., Kurzbauer, R, Schneider, A., Reifinger, M. and Loupal, G. (1995). Mutation in a tumour suppressor gene p 53 in two feline fibrosarcomas. *Br. vet. J.* **151**, 707 - 713.

Schmidt, W.. Schweighofer, T., Herbst, E., Maass, G., Berger, M., Schilcher, F., Schaffner, G. and Birnstiel, M.L. (1995). Cancer vaccines: The interleukin 2 dosage effect.. *Proc. Natl. Acad. Sci. USA* **92**, 4711 - 4714.

Smrzka, O.W., Faé, I., Stöger, R., Kurzbauer, R., Fischer, G.F., Henn, T., Weith, A. and Barlow, D. (1995). Conservation of a maternal-specific methylation signal at the human IGF2R locus. *Human Molecular Genetics* **4**, 1945 - 1952.

Public Relations department

Public Relations officer Heidemarie HURTL

The Public Relations Office at the I.M.P. serves various different functions. Its main objective is to give the public an idea of the research areas that are covered by the Institute's working groups and to communicate the most interesting results to an audience beyond the scientific community. By doing so, we try to create an awareness of the role of basic research in general, especially with regard to future developments in medicine.

The I.M.P. is regularly visited by groups of pupils, mainly through the initiative of biology and chemistry-teachers. The students generally show great interest in the research environment and in topics such as the mechanisms of cancer development or the principles of genetic engineering. With the support of the scientists, it is usually possible to visit some of the labs, the favorites being the Service Department and the Computer Group.

Apart from school-classes, visitors to the I.M.P. in 1995 also included journalism students from the Vienna University, a group of journalists from Hong Kong, biology- and chemistry-teachers from the Pedagogical Institute Salzburg, and numerous visitors from our mother-company Boehringer Ingelheim and subsidiaries.

One of the biggest PR-events in 1995 was Boehringer Ingelheim's International R&D Press Conference, which was held at the I.M.P. in May. It was quite a challege to organize not only the Press Conference itself but to guide large groups of journalists and their hosts through the institute and its labs. The success of the day was only possible with the help of all I.M.P. colleagues.

Also in May, the I.M.P.'s meanwhile traditional Spring Conference was held at Vienna's Austria Center. Although a mainly scientific event, the conference "Interfaces Between Cancer and Development" was very well received by the media and covered by newspapers, magazines, TV and radio.

Another major event in 1995 was the opening and "baptizing"-ceremony of our new library in June. To honor the Nobel Prize-winning biochemist Max Perutz, who was born and educated in Vienna, the Vienna Biocenter decided to name the library after him. The celebration was attended by Max Perutz and his wife, as well as numerous guests from politics, science and industry. The Austrian Science Minister used the opportunity to bestow upon Max Perutz a high award by the Republic of Austria for his outstanding services to the country.

From time to time, the I.M.P. is host to events organized by other institutions such as the Austrian Industrial Research Promotion Fund. Following an exhibition of cartoons related to research which depicted mostly male scientists, and the realization of female awareness at the institute, the fund organized a symposium on the role of women in science. A number of women scientists from various different fields were invited to talk about their personal experiences, triggering a lively discussion with the audience which was subsequently echoed by the media.

Apart from organizing visits and other events, the PR-office publishes press-releases, articles and brochures, arranges interviews with scientists, maintains a video- and photo-archive and generally provides science-related information to journalists wherever possible.

Diploma- and PhD-theses of I.M.P. students in 1995

Christoph Barnas (Group Andreas Weith)
"Molecular genetic analysis of neuroblastoma". PhD thesis, submitted to the University of Vienna.

Christian Dahmann (Group Kim Nasmyth)
"Mechanisms that restrict DNA replication to once per cell cycle in the budding yeast Saccharomyces cerevisiae". PhD thesis, submitted to the University of Vienna.

Petra Dörfler (Group Meinrad Busslinger)
"Cloning and characterization of the B-cell-specific transcription factor BSAP (Pax-5)". PhD thesis, submitted to the University of Vienna.

Helga Edelmann (Group Lisa Ballou)
"Regulation of p70/p85 S6 kinase". PhD thesis, submitted to the University of Vienna.

Irene Fialka (Group Hartmut Beug)
"The role of protooncogenic transcription factors in the modulation of mammary epithelial cell polarity". PhD thesis, submitted to the University of Vienna.

Walter Lerchner (Group Denise Barlow)
"Allele specific expression pattern of the imprinted Igf2r/Mpr300 locus during mouse embryonic development". Diploma thesis, submitted to the University of Vienna.

Birgit Panzenböck (Group Matt Cotten)
"The Role of the Adenovirus Protease in NF-κB Activation". Diploma thesis, submitted to the University of Vienna.

Karl Martin Schwarz (Group Hartmut Beug)
"Tetracycline-controlled gene expression based on retroviral vectors: Analysis of the function of BCR-Abl and mutated c-Abl oncoproteins in hematopoietic cells". Diploma thesis, submitted to the University of Vienna.

Norbert Schweifer (Group Denise Barlow)
"Positional cloning and analysis of the candidate region containing the implantation specific t^{w73} gene in *Mus musculus*". PhD thesis, submitted to the University of Vienna.

Robert Siegmund (Group Kim Nasmyth)
"Analysis of the transcription factors SBF and MBF required for entry into the cell cycle in Saccharomyces cerevisiae". PhD thesis, submitted to the University of Vienna.

Awards and nominations in 1995

Denise Barlow
was appointed EMBO-member.

Thomas Böhm
won the Prize for the Advancement of Medical Research in Austria, awarded by the Hoechst-Foundation, for the paper "The B-Type Cyclin Kinase Inhibitor $p40^{SIC1}$ Controls the G1 to S Transition in S. cerevisiae"

Kim Nasmyth
was awarded the Silver Medal by the Federation of the European Biochemical Societies (FEBS) and was appointed Honorary Full Professor at the University of Vienna.

Erwin F. Wagner
was awarded the Alois Sonnleitner Prize from the Austrian Academy of Sciences for outstanding achievements in developmental and tumor biology.

Andreas Weith
was appointed HUGO-member and Chromosome 1 editor.

I.M.P./Biocenter seminar list 1995

JANUARY

13.01.95
DAVID PORTEOUS (Edinburgh)
"Schizophrenia and cystic fibrosis: Genomics and gene therapy to the rescue?"
Host: Max L. Birnstiel

FEBRUARY

02.02.95
KAY DAVIES (University of Oxford)
"Molecular analysis of muscular dystrophy"
Host: Denise Barlow

09.02.95
WALTER GEHRING (Basel)
"*Eyeless*, the master control gene for eye morphogenesis"
Host: Meinrad Busslinger

23.02.95
PATRICK BÄUERLE (Freiburg)
"NF-κB: a transcription factor specialized in responding to pathogens"
Host: Matt Cotten

MARCH

02.03.95
RON LASKEY (Cambridge)
"Regulatory roles of the nuclear membrane"
Host: Erhard Wintersberger

09.03.95
JEAN-PIERRE KRAEHENBUHL (Lausanne)
"The challenge of mucosal immunization"
Host: Ernst Küchler

10.03.95
RIK DERYNCK (UCSF)
"The complexity of TGF-β biology and the role of EGF-receptor in mice"
Host: Erwin F. Wagner

15.03.95
DONALD MORTON (John Wayne Cancer Inst.)
"Melanoma cell vaccine"
Host: Max L. Birnstiel

20.03.95
SIDNEY ALTMANN (Yale)
"RNase P and its substrates: Prospects for gene therapy"
Host: Alexander von Gabain

22.03.95
TANJA NARANDA (Davis)
"RNA binding proteins in eukaryotic translational initiation"
Hosts: Hartmut Beug/Ernst Müllner

23.03.95
JOAN MASSAGUÉ (Sloan Kettering, NY)
"Antimitogenic signaling by TGF-β"
Host: Kim Nasmyth

24.03.95
REED WICKNER (NIH, Bethesda)
"[PSI] and [URE3] are prions of yeast: genetic evidence"
Host: Tim Skern

28.03.95
GIULIO SUPERTI-FURGA (EMBL)
"Regulation of the Src and Abl tyrosine kinases"
Host: Denise Barlow

30.03.95
FOTIS KAFATOS (EMBL)
"Transcription factors and post-embryonic development in *Drosophila*"
Host: Max L. Birnstiel

31.03.95
AARON MITCHELL (Columbia University)
"Unusual regulators that govern meiotic gene expression in yeast"
Host: Dieter Schweizer

APRIL

05.04.95
RANDY SCHEKMAN (Berkeley)
"Protein sorting along the secretory pathway"
Host: Karl Kuchler

06.04.95
DAN GOTTSCHLING (Chicago)
"Telomeric position effect in yeast: the dynamics of chromosomes in gene expression"
Host: Thomas Jenuwein

20.04.95
PHILIP COHEN (Dundee)
"Dissection of protein kinase cascades involved in cellular responses to growth factors and to stress"
Host: Manuela Baccarini

27.04.95
DAVID BARONI (Milan)
"Cyclic AMP can control different steps of the yeast cell cycle"
Host: Helmut Ruis

28.04.95
ROBERT BENEZRA (Memorial Sloan-Kettering, NY)
"Control of helix-loop-helix protein activity"
Host: Kim Nasmyth

MAY

05.05.95

STEVE JACKSON (Cambridge)
"DNA-dependent protein kinase; deciphering its roles in transcription,V(D)J recombination, and DNA repair."
Host: Erwin F. Wagner

17.05.95

DIRK BOHMANN (EMBL)
"Biochemistry and genetics of the transcription factor Jun"
Host: Peter Steinlein

JUNE

01.06.95

ANDY McMAHON (Harvard)
"Regulation of CNS pattern by cell signaling"
Host: Meinrad Busslinger

02.06.95

PAUL NURSE (ICRF, London)
"Controlling the cell cycle in fission yeast"
Host: Kim Nasmyth

08.06.95

AXEL ULLRICH (Martinsried)
"Target-directed antiangiogenic therapy of glioblastoma"
Host: Erwin F. Wagner

14.06.95

CHRISTIAN HAASS (Univ. Mannheim)
"Molecular and cellular biology of Alzheimer´s disease"
Hosts: Frank Hilberg/Erwin F. Wagner

20.06.95

DAVID ROBINSON (Wessex Regional Genetics Lab., UK)
"Uniparental disomy and transient neonatal diabetes mellitus in humans"
Host: Denise Barlow

22.06.95

HERMANN BUJARD (Heidelberg)
"Controlling individual gene activities: Mechanisms and applications"
Host: Erwin F. Wagner

JULY

13.07.95

JANET ROSSANT (Toronto)
"Genetic control of early mouse development"
Host: Zhao-Qi Wang

14.07.95

RUDOLF JAENISCH (Whitehead Inst., Boston)
"Role of DNA methylation in vertebrate development"
Host: Erwin F. Wagner

20.07.95
MICHAEL SCHLEICHER (Munich)
"Molecular dynamics of the actin cytoskeleton in amoeboid cells"
Host: Gerhard Wiche

27.07.95
ALAN HINNEBUSCH (NICHD, Bethesda)
"Gene-specific translational control of transcriptional activator GCN4 by modulation of general protein synthesis factors"
Host: Kim Nasmyth

AUGUST

29.08.95
SELINA CHEN-KIANG (Mount Sinai)
"Molecular crosstalk between Stat, bZIP proteins and pRB in cytokine signaling"
Host: Kim Nasmyth

SEPTEMBER

07.09.95
TOMAS LINDAHL (ICRF, London)
"Human enzymes acting as DNA strand interruptions"
Host: Zhao-Qi Wang

08.09.95
UTTAM SURANA (Singapore)
"Revisiting CDC28"
Host: Gustav Ammerer

13.09.95
MICHAEL PEPPER (Geneva)
"Angiogenesis: a balance between positive and negative regulators"
Host: Erwin F. Wagner

14.09.95
PASCAL HAFFTER (Tübingen)
"The identification of genes with unique and essential functions in the development of the Zebrafish"
Host: Max L. Birnstiel

15.09.95
ALCIDE BARBERIS (Milan)
"Contact with a component of the RNA pol II holoenzyme suffices for gene activation"
Host: Meinrad Busslinger

19.09.95
JOACHIM LINGNER (HHMI, Boulder, Co)
"Telomerase and the art of chromosome replication"
Host: Max L. Birnstiel

19.09.95
EDWARD E. SCHMIDT (Univ. Geneva)
"Cell type-specific regulation of and by the basal transcription machinery"
Host: Max L. Birnstiel

20.09.95
URSULA KLINGMÜLLER (Whitehead Inst.)
"Signal transduction through the Erythropoietin-Receptor"
Host: Max L. Birnstiel

20.09.95
JAN MICHAEL PETERS (Harvard)
"Cyclin degradation in mitosis"
Host: Max L. Birnstiel

21.09.95
ROGIER VERSTEEG (Univ. Amsterdam)
"A modifier of methylation for class I HLA genes maps to chromosome 1p35-36 and is deleted in neuroblastomas"
Host: Denise Barlow

21.09.95
MICHAEL ROSBASH (HHMI, Waltham, Mass.)
"Circadian rhythms in Drosophila"
Host: Kim Nasmyth

OCTOBER

16.10.95
MITSUAKI YOSHIDA (University of Tokyo)
"Molecular interaction of the oncoprotein of HTLV-1 and host cells for the pathogenesis"
Host: Max L. Birnstiel

18.10.95
HIROAKI KIYOKAWA (Sloan Kettering)
"Control of mammalian cell growth and differentiation by cyclin-dependent kinases"
Hosts: Hartmut Beug/Ernst Müllner

19.10.95
MARTIN RAFF (London)
"Programmed cell death"
Host: Kim Nasmyth

25.10.95
MAREK LOS (DKFZ, Heidelberg)
"The APO-1/Fas signal transduction pathway"
Host: Zhao-Qi Wang

NOVEMBER

07.11.95
DEEPAK BASTIA (Durham, NC)
"Crystal structure and mechanism of action of a replication terminator protein"
Host: Kim Nasmyth

09.11.95
HAMILTON SMITH (Baltimore)
"Comparative genomics between *Haemophilus influenzae* and *Mycoplasma*"
Host: Max L. Birnstiel

10.11.95
SALLY CROSS (Edinburgh Univ.)
"Libraries of CpG islands: a tool for mapping and cloning genes"
Host: Denise Barlow

16.11.95
NAHUM SONENBERG (McGill Univ. Montreal)
"Translation initiation factors in control of gene expression, cell growth and tumorigenesis"
Host: Ernst Küchler

22.11.95
JOEL EISSENBERG (St. Louis)
"Epigenetic regulation in Drosophila: a conspiracy of silence"
Host: Thomas Jenuwein

23.11.95
KEN HOLMES (Heidelberg)
"The molecular basis of muscular contraction"
Host: Erhard Wintersberger

DECEMBER

07.12.95
YEN CHOO (MRC Cambridge, UK)
"Understanding and exploiting sequence-specific DNA-binding by zinc fingers"
Host: Max L. Birnstiel

12.12.95
ANDREAS HEDER (MD Anderson Cancer Center, Houston)
"Transactivation of HIV-TAT *in vitro*"
Host: Thomas Jenuwein

I.M.P. Patent applications 1995

CASE 14/029

"Method for introducing foreign material into higher eukaryotic cells"
Inventors: A. Baker, S. Chiocca, M. Cotten

Addition CASE 14/028

"Synthesis of a novel metal-chelator for the use in chromatography and biosensor-based applications"
Inventors: P. Steinlein, W. Zauner, B. Habermann

Addition CASE 14/025

"Method for producing and cultivating erythroid precursor cells"
Inventors: H. Beug, O. Wessely, M. van Lindern

Addition CASE 14/027

"Method for producing viral vectors"
Inventors: H. Lehrmann, B. Panzenböck, M. Cotten

CASE 14/028

"Synthesis of a novel metal-chelator for the use in affinity-chromatography in biosensor-based applications"
Inventors: P. Steinlein, W. Zauner

CASE 14/030

"Method to overcome immune-tolerance against unknown tumor-antigens using a genetically modified cancer vaccine"
Inventor: T. Schweighoffer

CASE 14/031

"Mammalian chromatin regulator genes"
Inventors: T. Jenuwein, G. Laible

CASE 14/032

"Method for generating potent anti-tumor immunity"
Inventors: M. Birnstiel, W. Schmidt, P. Steinlein, T. Schweighoffer

I.M.P. Publications 1995

1. Barlow, D.P. (1995). Gametic imprinting in mammals. *Science* **270**, 1610-1613.

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6. Beug, H., Steinlein, P., Bartunek, P. and Hayman, M.J. (1995). Avian hematopoietic cell culture: *In vitro* model systems to study oncogenic transformation of hematopoietic cells. In: *Methods in Enzymology* **254**, Academic Press, Inc. pp. 41-76.

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12. Chichlia, K., Moldenhauer, G., Daniel, P.T., Busslinger, M., Gazzolo, L., Schirrmacher, V. and Khazaie, K. (1995). Immediate effects of reversible HTLV-1 tax function: T-cell activation and apoptosis. *Oncogene* **10**, 269-277.

13. Christofori, G., Naik, P. and Hanahan, D. (1995). Deregulation of both imprinted and expressed alleles of the insulin-like growth factor 2 gene during ß-cell tumorigenesis. *Nature Genetics* **10**, 196-201.

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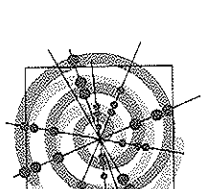


I.M.P.

Research Institute of Molecular Pathology

1996 Ph.D. Program in:

Cancer, Cell Cycle, Cellular Biology, Development, Gene Expression,
Gene Therapy, Microbial Genetics
(*Competitive stipends*)

Hartmut Beug, Meinrad Busslinger, Gerhard Christofori, Matt Cotten,
Lukas Huber, Thomas Jenuwein, Kim Nasmyth, Erwin F. Wagner, Andreas Weith

For information write or fax before January 18, 1996, to

Prof. Helmut Ruis
Vienna Biocenter, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria
Fax: ++43 1 799 5272

Deadline for return of application forms:
February 10, 1996

6TH IMP SPRING CONFERENCE ON

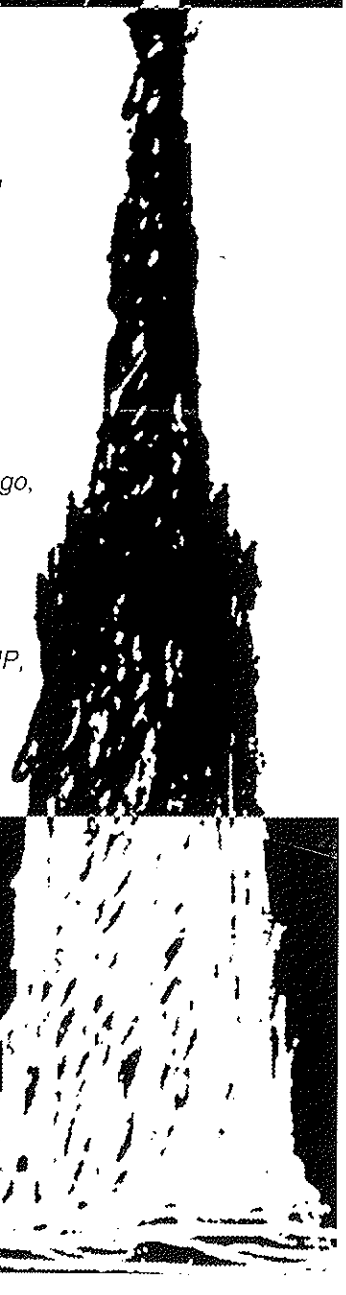
„INTERFACES BETWEEN CANCER & DEVELOPMENT“

IMP

11 - 13 MAY 1995, VIENNA

Austria Center

Organised by: Erwin F. Wagner (IMP) and Douglas Hanahan (UCSF)



Sessions on:	chaired by:
- CELL DIVISION AND DEATH	**G. Evan** *ICRF*
- SIGNALLING	**J. Schlessinger** *NYU*
- TUMOR SUPPRESSOR GENES	**E. Harlow** *Harvard*
- ANGIOGENESIS and METASTASIS	**J. Folkman** *Harvard*
- GENETIC INSTABILITY	**D. Housman** *MIT*
- MULTISTAGE ONCOGENESIS	**S. Cory** *WEHI*

Speakers include:

D. Baltimore *MIT*, D. Beach *CSH*, A. Berns *Amsterdam*, N. Bouck *Chicago*, D. Cheresh *La Jolla*, S. Cory *WEHI*, G. Evan *ICRF*, J. Folkman *Harvard*, C. Greider *CSH*, D.Hanahan *UCSF*, E. Harlow *Harvard*, J. Hoeijmakers *Rotterdam*, D. Housman *MIT*, M. Karin *UCSD*, D. Lane *Dundee*, F. McCormick *Onyx*, S. Nagata *Osaka*, K. Nasmyth *IMP*, O. Pereira-Smith *Houston*, P. Polakis *Onyx*, B. Reid *Seattle*, W. Risau *MPI Bad Nauheim*, J. Schlessinger *NYU*

Abstracts for poster presentations are invited; a few will be selected for oral presentations.

Registration/abstract deadline is **28 February 1995**
Registration fee is **1500** Austrian Schillings; **500** for students or participants from Eastern Europe.

For registration material please contact:

Diane Turner, *Meetings Coordinator*
IMP, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria
Tel: +43-1-797 30/451 FAX: +43-1-798 71 53