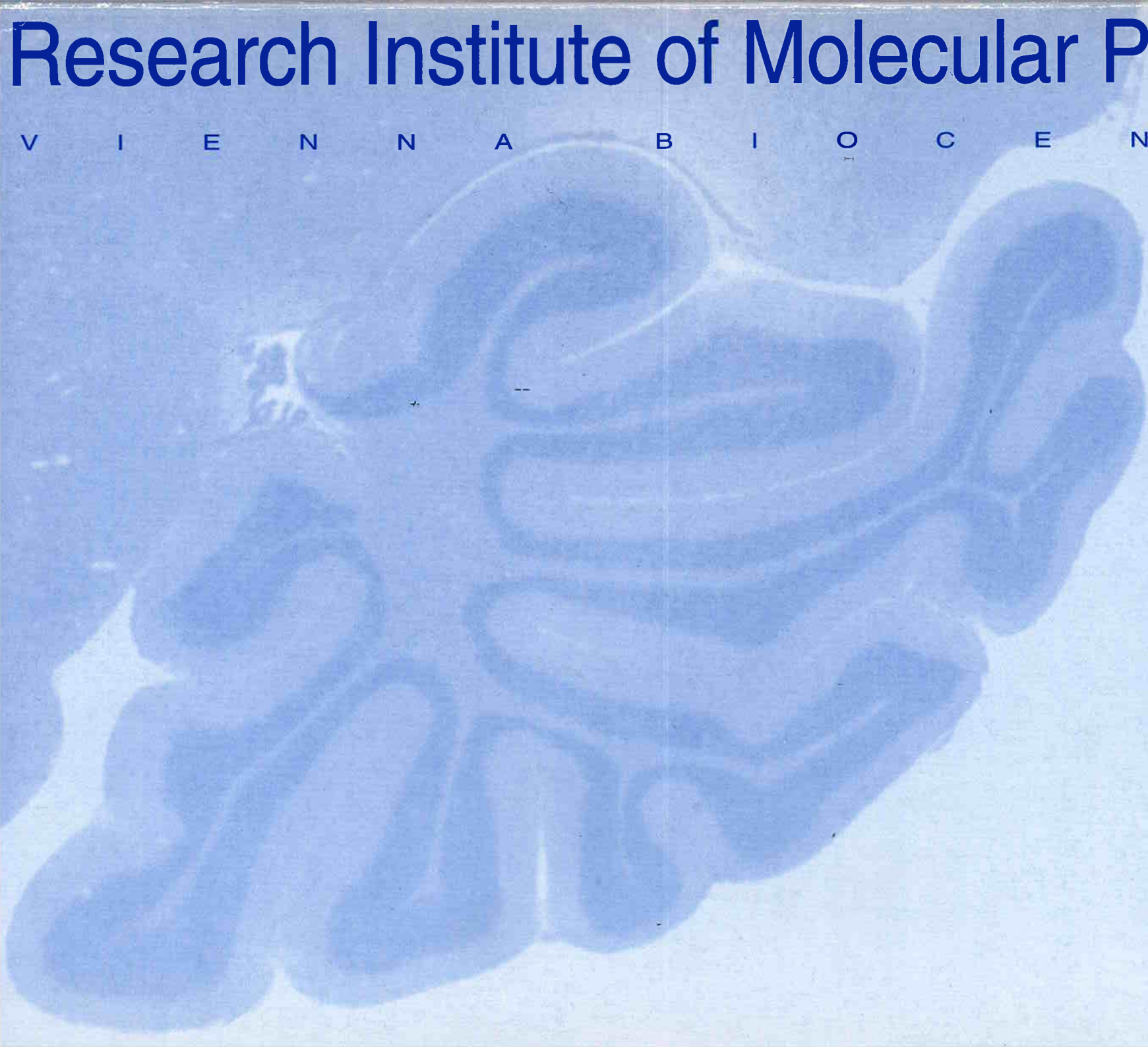
Research Institute of Molecular Pathology

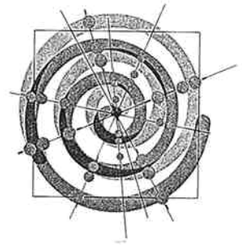
V I E N N A B I O C E N T E R



I M P
1994

Cover: Abnormal morphogenesis of the posterior midbrain and anterior celebellum in Pax-5-deficient mice.
(Front cover: mutant brain, back cover: wild type brain. For details see report group Busslinger.)

Scientific Report
1994



Research Institute of Molecular Pathology (I.M.P.)
Dr. Bohr-Gasse 7
A-1030 Vienna
Austria
Tel.: (+43 1) 797 30
Fax: (+43 1) 798 71 53

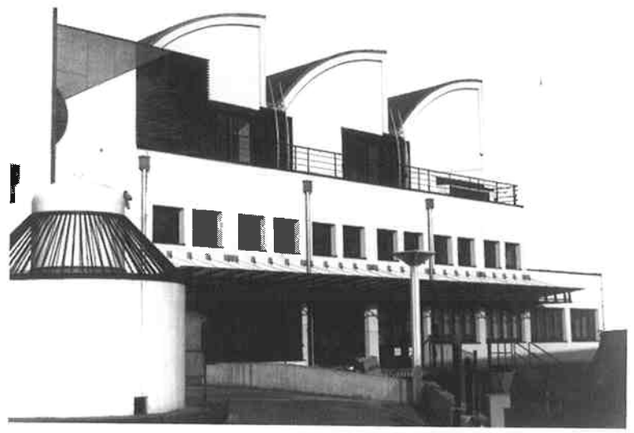
Contents

Impressum:

Edited by: I.M.P.
Dr. Bohr-Gasse 7, A-1030 Vienna, phone: (+43 1) 797 30
Managing Director: Prof. Dr. Max L. Birnstiel
Administrative Director: Dr. Nikolaus Zacherl
Coordination: Heidemarie Hurtl
Printed by: Agens-Werk Geyer + Reisser, 1050 Vienna
Cover photo: Pavel Urbánek/ Hannes Tkadletz, I.M.P.

Introduction

At the inception of the IMP the long term goal of the Research Institute was seen as the development of new concepts, in the hope that this would lead to the identification of potentially useful products for commercial application in the therapeutic and diagnostic areas. The developments in 1994 have provided an important "proof of principle" for the IMP-Boehringer alliance in that a series of molecular diagnostic probes of a chromosomal region harbouring the neuroblastoma tumor suppressor gene were submitted for patenting by Andreas Weith's group. In addition, the adenovirus-enhanced-transferrin-receptor-mediated gene transfer technique (the "IMP transfection system", for short) found its first potential medical application in the generation of whole-cell tumor vaccines. Indeed, a clinical protocol for the application of these tumor vaccines has been written by Prof G. Stingl of the Vienna University, Dr. K. Zatloukal of the Graz University and the staff of Bender (Vienna), another subsidiary of Boehringer Ingelheim. The protocol, based on the copious preclinial data from the Birnstiel group, passed the Austrian Arzneimittelbeirat (the equivalent of the American FDA) and the Ethical Commission of Vienna University in the fall of 1994. The transfection compounds required for implementing the tumor vaccines were prepared according to GMP by Bender under the supervision of Ernst Wagner, former group leader of the IMP. Phase I patient trials began in December 1994.



With the generous financial help of the City of Vienna the IMP was able to expand into adjacent shed halls and to gain an additional space of 2700 m² (see insert). The space has been used for an up to date spacious library run jointly with the University departments abutting the IMP, badly needed offices for post docs and students, a computerized supermarket-style store-room for supplies, a small extension of the animal house, and a new workshop, as well as three small appartments, much sought-after amenities for visiting scientists.

There has been some turnover amongst the groupleaders in that Ernst Wagner took over the GMP production of tumor vaccines at Bender. Gerhard Christofori, coming from Doug Hanahan's group at UCSF, joined the IMP in March 1994. He replaces Tillman Schuster who left the IMP with a view to taking up a professorship at the University of Würzburg (Germany). Christofori brings new and important areas of research, angiogenesis and the study of experimental insulinomas, to the Institute.

The IMP has now reached a size which we must consider final for some time to come. Today there are 12 teamleaders, 27 postdocs, 30 Ph.D. students, 25 technicians and a supporting cast of 38 in the various services such as workshop, media kitchen, service department library, stores and administration etc., giving a total of 132 IMP employees.

Members of the IMP received several honors. Thus León Dirik, Ph.D.student in Kim Nasmyth's group was awarded Schepken's Prize of Genetics by the Belgian Royal Academy of Sciences. Senior Scientist Erwin Wagner was appointed a.o. Professor at the Vienna University, and the Managing Director received the Wilhelm Exner Medal from the Austrian Gewerbeverein.

Productivity of the IMP was high with 9 patent applications and 102 papers having appeared or having been submitted in 1994. The Scientific Advisory Board composed of Profs. Michael Bishop (Chair), Nick Hastie, Jeff Schatz, Peter Swetly, Tada Tanigutchi and Robert Weinberg was joined by Prof. Christoph Hohbach, Research Director of Boehringer Ingelheim International, thus ensuring close contact between the IMP and its sponsor.

Max L. Birnstiel
Managing Director
Vienna, January 1995

Tumor Vaccines

Managing Director	Max L. BIRNSTIEL
Postdoc	Gerhard MAASS
Postdoc	Walter SCHMIDT
Postdoc	Tamás SCHWEIGHOFFER
Laboratory technician	Manfred BERGER
Laboratory technician	Elke HERBST
Laboratory technician	Helen KIRLAPPOS

in collaboration with:
Norma Howells, Animal House, I.M.P.;
Ernst Wagner and Michael Buschle, Bender + Co. Ges.m.b.H., Vienna;
Achim Schneeberger, Frieder Koszik and Georg Stingl, Dept. of Dermatology, Div. Imm. All. and Infect. Dis., Univ. of Vienna, Med. School;
Franz Schilcher, Institute of Pathology and Forensic Veterinary Medicine, and Cornelia Gabler, Institute of Nutrition,University of Veterinary Medicine, Vienna.

Introduction

During the past year we have extended our previous studies on the generation of whole cell vaccines capable of rejecting live tumor cells (in both prophylactic and therapeutic mouse models; see Scientific Report 1993) with a view of providing preclinical information necessary to produce a clinical protocol for the treatment of melanoma patients. Such a protocol was compiled by Prof. Georg Stingl (Univ. Clinic of Dermatology in Vienna) and Dr. Kurt Zatloukal (Institute of Pathology in Graz). As the whole cell vaccination procedure now enters the clinical phase, our I.M.P. group is expanding the preclinical studies to further optimize the vaccination effect. In addition, our group is extending these studies to include other mouse model systems and, most importantly, to investigate the basic mechanisms underlying the generation of the immune response which leads to rejection of live tumor cells.

Cytokine dosage impact on cancer vaccine efficacy

Walter Schmidt, Tamás Schweighoffer, Elke Herbst, Gerhard Maass, Manfred Berger and Franz Schilcher

One important question for the successful application of cancer vaccines, genetically engineered to secrete cytokines, is related to the cytokine dosage of the vaccines. We have addressed this question in the murine M-3 melanoma model and studied the dosage effects of two cytokines, interleukin-2 and GM-CSF. The vaccine consisted of M-3 tumor cells, transfected with murine IL-2 or GM-CSF expression vectors using the I.M.P. **a**denov**i**rus-**e**nhanced receptor-mediated **t**ransferrinfection (AVET) procedure. The transfected cells were irradiated prior to inoculation into syngeneic DBA/2 mice. 10^5 cells were injected per mouse and each animal received two vaccinations at an interval of one week. One week after the last immunization the mice were challenged with $3x10^5$ viable tumor cells which in untreated animals grow out to solid tumors within 10-14 days. We engineered M-3 tumor cells to secrete 21 different levels of IL-2. The in vitro IL-2 expression ranged from 0.01 to 7,500 units/10^5 cells and day. Control groups received the same number of either irradiated or mock-DNA transfected and irradiated tumor cells.

We find that the vaccination efficiency is significantly dependent upon the IL-2 dosage produced by the vaccine (**Fig. 1**). The best protection against subsequent challenge is achieved when animals are immunized with vaccines producing medium IL-2 levels of 1,000 to 2,000 units/10^5 cells, seven out of eight animals being protected from tumor take. A stepwise decrease of IL-2 production is accompanied by a corresponding decline in the number of protected animals indicating imperfect anti-tumor immunity. Below the expression of 10 units IL-2/10^5 cells x day the efficiency approaches the background protection of control groups, being approximately 15% when mock-DNA transfected, irradiated cells are used (22 of 26 animals develop a tumor) or 11% with irradiated cells alone (46 of 52 tumor positive).

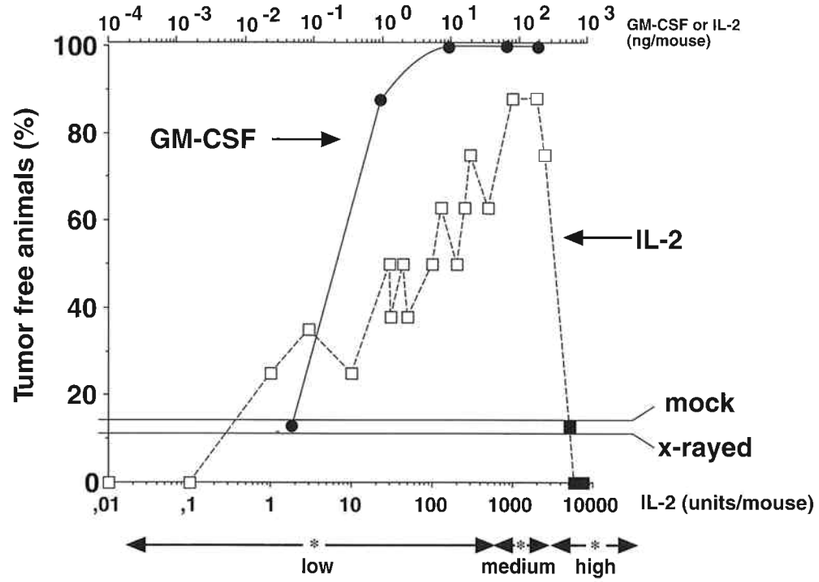


Fig. 1: IL-2 and GM-CSF dosage curves

Surprisingly, in the high interleukin-2 production range (5,000 units/10^5 cells and day or more), no significant systemic immunity can be generated. Application of 5,000 units IL-2/10^5 cells allows the growth of parental M-3 cells in seven out of eight challenged animals and at higher dosages in eight out of eight animals. This contrasts with the response obtained with GM-CSF releasing M-3 cells. Immunity is already high at moderate secretion and reaches a plateau at maximally obtainable GM-CSF production (Schmidt *et al.*, 1994).

We identified the failure to generate tumor-specific cytotoxic T cells (CTLs) as the reason for the overshoot phenomenon observed in the case of high IL-2 secretion (**Fig. 2**). CTLs have been identified as the major effector cells for antitumor immunity and their presence is essential for the destruction of the challenging tumor cells. Other consequences from the injection of high level IL-2 secreting M-3 cells are not detectable, and we also do not see signs of vascular leakage syndrome which is a side effect of high-dose recombinant IL-2 therapy. Thus, serum transaminase levels, indicators of liver damage are not altered, histological analysis reveals the absence of any liver tissue abnormality and we do not see tissue oedema in any of the organs (not shown).

We find that the failure of high IL-2 secreting M-3 cells to elicit an anti-tumor effect correlates with the appearance of low, short-term, systemic blood levels of IL-2. When protective levels of 2,000 units vaccine-expressed IL-2 are injected at three sites of an animal with a total expression of 6,000 units/mouse, the same detrimental effect is obtained as with a vaccine expressing 6,000 units IL-2 applied as a single inoculum (**Tab. 1**).

The immunization site itself, a location of possible interference with the generation of long-lasting anti-tumor immunity, is apparently not affected by high dosage IL-2 producing vaccines. Cellular infiltrates, mainly consisting of macrophages accompanied by granulocytes and NK-cells, and secondary cytokine expression patterns at the injection site (IL-1, IL-6 and

Total amount of IL-2 (units/mouse)	No. of injection sites	IL-2 (units)/site	Tumor free animals
-	1	-	0/10
2000	1	2000	7/8
6000	1	6000	0/8
6000	3	2000	2/8
2000	3	670	7/8

Tab. 1: Comparison of local and systemic IL-2 effects

IL-10) are identical after vaccination with medium or high level IL-2 producers (data not shown). These data suggest that the failure to generate tumor-specific CTLs at high IL-2 secretion values may be a late event in the process leading to the appearance of such CTLs.

Our results from the M-3 melanoma model demonstrate that IL-2 and GM-CSF secreting cancer vaccines can generate a powerful antitumor protection. For IL-2 there is a sharp dosage optimum for best efficacy, whereas the GM-CSF production seems to be less critical.

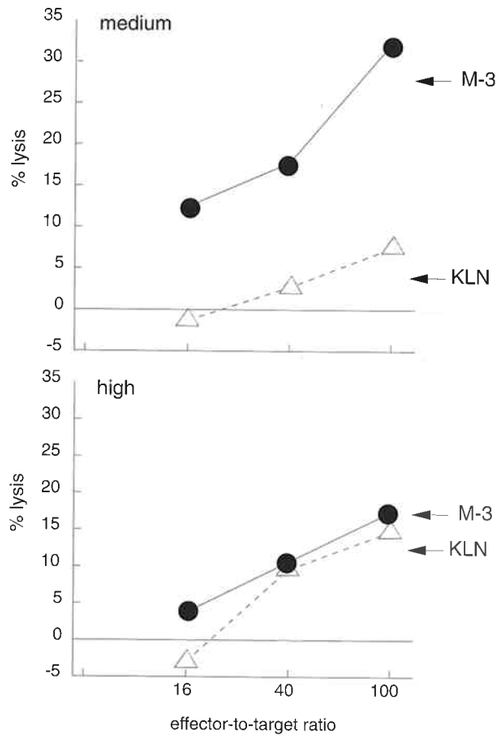


Fig. 2: Lysis of M-3 cells by spleen cells is specific after immunization with transfected M-3 cells expressing medium levels of IL-2, but specificity is lost if the vaccine produces high levels of IL-2

Murine colon cancer CT-26

Walter Schmidt and Elke Herbst

To further examine the potency of cytokine releasing cancer vaccines, we tested IL-2 or GM-CSF producing tumor cells in the CT-26 colon carcinoma model and also included a third cytokine, IFN-γ. Irradiated (x-ray) and irradiated, mock DNA transfected (mock) cells were used as controls. The experimental design was the same as in the M-3 model: two vaccinations with 1x10^5 irradiated, cytokine gene transfected cells followed by a challenge with 3x10^5 viable tumor cells one week later. Cells were modified to express three different levels of the respective cytokine gene, referred to as high, medium and low. For IL-2, best vaccination was obtained with the high expression level (4,000 units/1 x 10^5 cells/day) which provided protection for 7/8 animals (**Fig. 3**). With the medium level (800 units/10^5 cells/day) protection rate dropped to 50%, and with the low expression value to 25%, which is in the range of the control groups (x-ray: 20%; mock: 25% protection). These results demonstrate again the dosage impact of interleukin-2 on the potency of the cancer vaccine, although the absolute amount necessary for best protection in the CT-26 colon carcinoma is different from that found in the M-3 model. For GM-CSF, the high level (200 ng/mouse x day) and medium level groups (20 ng/mouse x day) were protected with similar efficiencies, whereas with the low level group (2 ng/mouse x day) only background protection was obtained. For IFN-γ, the protection rate was only slightly better than with the controls, and no dosage effect was observed.

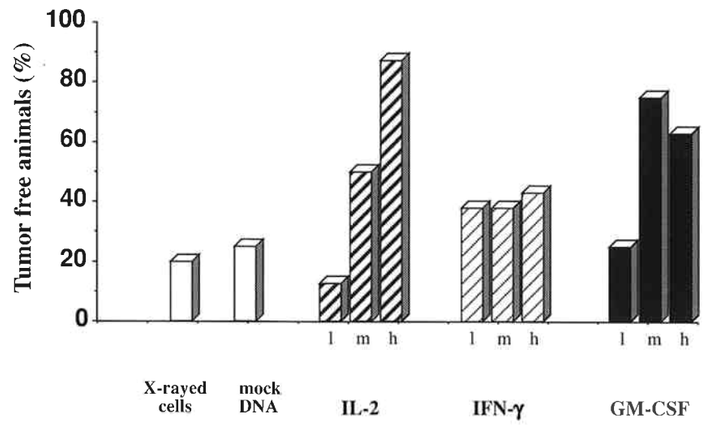


Fig. 3: Vaccination in the CT-26 colon carcinoma model

Cancer vaccines in therapeutic mouse tumor models

Walter Schmidt, Cornelia Gabler, Elke Herbst, Norma Howells and Helen Kirlappos

The results described in the previous sections demonstrate the impressive efficacy of cytokine producing cancer vaccines in generating anti-tumor immunity. In these experiments, the vaccine was always applied prior to tumor challenge (prophylactic model). In the human situation, however, the task is quite different since patients may suffer from inaccessible organ metastases which are established before a possible treatment can be applied. In order to study vaccines in a scenario more closely related to the situation in patients, we analyzed the anti-metastatic capacity of the IL-2, IFN-γ and GM-CSF-producing CT 26 cells in a therapeutic experimental design. 10^4 viable CT 26 cells were injected i.v. to generate micrometastases in the lung. After two days, when the metastases can be expected to have settled and started to grow, vaccination with 1x10^5 transfected, irradiated cells was started. The vaccination was repeated once after seven days and on day 21 animals were analyzed for lung metastases. All three vaccines,

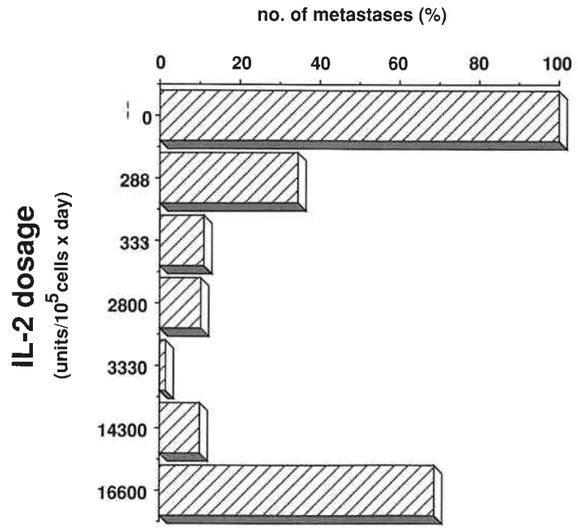


Fig. 4: IL-2 dosage dependent reduction of B16-F10 metastases

producing IL-2, GM-CSF or IFN-γ reduced the number of metastases by app. 50% compared to untreated animals, demonstrating that cytokine producing cancer vaccines can also affect pre-existing metastases (data not shown).

Our second target for the application of cancer vaccines in a therapeutic model are metastases of melanoma origin. Since the M-3 melanoma is a non-metastatic tumor, we chose the highly metastatic B16-F10 cell line for our therapeutic studies. When injected intravenously, B16-F10 cells preferentially form primary metastases in the lung but are thought to form secondary lymph node metastases. A first set of experiments, demonstrating an increased number of tumor free animals when the vaccines produced IL-2, GM-CSF or IFN-γ compared to non cytokine producers has been reported in the 1993 I.M.P. Scientific Report.

Unlike the prophylactic M-3 model, the interleukin-2 dosage optimum for significant reduction of lung metastases was much broader (**Fig. 4**). To demonstrate the curative benefit of cancer vaccine treatment on metastatic disease we investigated the survival of mice suffering from both primary intradermal B16-F10 tumor and lung metastases. The primary tumor was removed by surgery whereas lung metastases were treated with cytokine releasing vaccines. Three month survival was taken as the criterion for successful treatment (**Tab. 2**). In all control groups which received either no treatment, untransfected, irradiated or mock-DNA transfected, irradiated cells, 7/8 animals died from the metastases. In the GM-CSF and IFN-γ groups, 4/8 the animals survived, and with the interleukin-2 secreting vaccine 5/8 animals were cured with intravenous and even 6/8 with subcutaneous treatment. This result demonstrates that tumor cells, genetically engineered to secrete cytokines, can vaccinate mice against pre-existing organ metastases.

no treatment	1/8	
vaccine	**application**	
	i.v.	s.c.
x-ray	-	1/8
mock	0/8	1/8
IL-2	5/8	6/8
IFN-γ	4/8	0/8
GM-CSF	4/8	-

Tab. 2: Cure of C57/BL6J mice of preexisting metastases

Novel strategies for tumor vaccines based on redirected memory response

Tamás Schweighoffer and Manfred Berger

Tumor cells engineered either to secrete large quantities of various cytokines such as IL-2 and GM-CSF, or to express costimulatory molecules were shown to elicit efficient host-anti-tumor responses in experimental animal models. We propose (see below) that generation of an effective anti-tumor response requires that macrophage-like cells are recruited to the vaccination site, they process and present tumor-antigen and finally prime naive T cells to become anti-tumor effector cells. In patients, however, who already have a considerable tumor burden, it may be exceptionally hard to accomplish this cascade of complex interactions: on one hand, the host is essentially tolerant against the tumor, on the other hand, also some of the general immune functions may be impaired. Consequently, a better approach might be to evoke an existing, well-defined memory-type cellular response and redirect it against genetically modified tumor cells that are used as immunogens. This way, pre-existing specific memory type T cells would access the immunogen which should result in immediate recognition and subsequently a faster and more predictable build-up of anti-tumor immunity. Careful selection of the target antigen is, however, necessary to achieve these goals.

Immunization with M. bovis BCG leaves behind a stable and long-lasting (in many cases lifelong) immunity. In humans, this protection can be characterized as a delayed-type hypersensitivity (DTH) reaction that is generated primarily by Th1-(inflammatory)-type T cells. This reactivity pattern has been observed in cases with competent host-anti-tumor reactions and in general seems to be a requisite for efficient anti-tumor responses. The 65-kDa heat shock protein hsp65 has been shown to be one of the several M. bovis-derived proteins that are capable of inducing the DTH reaction. We have constructed a truncated hybrid of the hsp65 protein, designated Heat1, that is suitable for expression in mammalian cells. The expressed protein was characterized upon transfection into COS cells by FACS analysis and Western blotting using a panel of monoclonal antibodies.

To test the idea whether a redirected pre-existing immune response could be mobilized against tumors, we first established active anti-BCG -immunity in DBA/2 mice. Immunized mice were then enrolled in two different tumor vaccine models. Throughout these experiments the mouse melanoma line M-3 was used; this cell line is efficiently transfected by AVET (**A**de-no**v**irus **e**nhanced receptor-mediated **t**ransfection) resulting in routinely high levels of Heat1 expression in about 25% of the cells.

Loss-of-tumorigenicity experiment: $1x10^5$ Heat1 expressing transfected M-3 melanoma cells were injected into 1.) naive, 2.) soluble recombinant hsp65 protein preimmunized or 3.) whole BCG preimmunized DBA/2 hosts, and tumor outgrowth monitored subsequently. While tumor development in soluble hsp65 preimmunized and naive hosts was similar to control animals (i.e. naive DBA/2 mice inoculated with wild-type M-3), most BCG-preimmunized mice remained tumor-free with only one animal showing retarded tumor outgrowth. Thus, Heat1 expressing tumor cells can efficiently be targeted by the appropriately primed host immune system. In contrast to the cytokine-secreting vaccine systems, T cells were detected in low but significant numbers at the site of injection by immunohistochemistry, supporting the idea that anti-BCG memory T cells participate in the initiation of the anti-tumor response.

Prophylactic experiment: DBA/2 mice were preimmunized with whole BCG and rested for 15 weeks. Two sc. immunizations of 10^5 pHeat1 transfected M-3 cells with one week interval were administered, and after an additional week mice were challenged with 10^5 live M-3 cells (100-fold tumorigenic dose) subcutaneously. Ten weeks after challenge, four out of 8 mice were tumor-free; the other four showed small black spots at the challenge site that did not substantially grow throughout the experiment, and did not otherwise impair the health of these mice. The cellular composition of this infiltrate is not clear yet.

These preliminary tests show that a pre-existing immune response can be redirected to control tumor growth. With over 40% of the world population vaccinated with BCG, there is hope that such a strategy would be widely applicable also for human therapy.

Three consecutive steps may be required for T-cell priming after IL-2 tumor vaccination

Gerhard Maass, Walter Schmidt, Frieder Koszik, Manfred Berger, Achim Schneeberger, Georg Stingl, Max L. Birnstiel and Tamás Schweighoffer

Having shown that $CD4^+$ and $CD8^-$ T-cells are required for the generation of a protective, long-lasting immunity induced by subcutaneous administration of IL-2 transfected, irradiated M-3 murine melanoma cells (Zatloukal *et al.*, 1994) we wanted to determine the site and mechanism(s) of T-cell sensitization. There are essentially two conflicting theories in the literature about this point. The first hypothesis posits that the tumor cells secreting cytokines act as antigen-presenting cells leading to T-cell activation at the vaccination site. More recently, it has been considered that antigen presentation may be based on classical APCs, followed by T-cell activation in the draining lymph node (Bannerji *et al.*, 1994).

In order to distinguish between these two possibilities we investigated the vaccination site of IL-2-secreting M-3 cells by means of immunohistology to identify the sequence of early events in the generation of the immune response. We found that there is a massive infiltration consisting of macrophages and granulocytes which eliminate the tumor bolus within 48 hours. No T-cells in significant numbers could be found at the vaccination site.

To confirm that T-cells need not be involved in the immunization site reactions, T-cell-incompetent nude mice were injected with irradiated, IL-2-secreting or non-secreting M-3 cells. The inocula of injected cells were scrutinized for invading immune cells and the data were compared with those obtained from immune-competent DBA/2 mice. Infiltrating cells quantitatively matched the vaccine infiltrate of immune-competent mice with macrophages accumulating rapidly in IL-2-secreting inocula. The only difference between athymic and euthymic mice was the magnitude of granulocyte response, the IL-2-expressing inoculum showing a 3-fold greater increase in granulocyte numbers in nude mice as compared to DBA/2 mice at 24 hours after injection. These tumor masses which secreted IL-2 were essentially eliminated at 48 hours in the nude mouse, a reaction almost identical to that in

the syngeneic, immune competent host. This is evidence that cytokine-secreting tumor cell masses can be rejected in a system devoid of T-cells, presumably by the non-specific immune response based on macrophages, NK-cells and granulocytes.

The rapid kinetics of vaccine elimination prompted us to start analysis of mRNA isolated from cells recovered as early as 6 hours after subcutaneous injection. Results are summarized in **Tab. 3a**. IL-2-secreting irradiated M-3 cells induced mRNA signals for IL-1β, IL-6, IL-10 and GM-CSF. This is a typical cytokine pattern for a macrophage-induced inflammatory immune response. By contrast, irradiated non-transfected M-3 in melanoma cells induced only weak IL-10 and the GM-CSF signals in the immune competent host. Plasmid-derived IL-2 production continued for approximately 48 hours post immunization, and the disappearance of IL-2 coincided with the loss of secondary cytokine response.

Similar experiments were performed in T-cell deficient nude mice: interestingly, the same cytokine pattern could be detected after immunization with IL-2-transfected M-3 cells. Therefore, the mRNAs coding for the cytokines IL-1β, IL-6, IL-10 and GM-CSF which were found to be induced in the previous experiments are unlikely to be of T-cell origin.

We therefore reasoned that draining lymph nodes might be the anatomic location of lymphocyte priming. Indeed, mRNAs of cytokines indicative of T-lymphocyte activation were detected by RT-PCR in the enlarged draining lymph nodes of DBA/2 mice immunized with IL-2 transfected tumor cells. In these animals, transcripts for the cytokines IL-4 and IFN-γ were detectable for a period of at least 5 days. This mRNA population could not be amplified from lymph node cells after immunization with non-transfected tumor cells or from naive mice (**Tab. 3b**). In addition, enhanced mRNA levels for IL-2, IL-2 receptor and CD69 (a T-cell activation marker) could be detected in lymph node cells after IL-2 immunization, which also argues for T-cell expansion in the draining lymph nodes.

Given these facts we hypothesize that macrophages eradicating the M-3 cells at the vaccination site may transfer tumor antigen(s) into the lymph node for presentation to T-cells. A variety of techniques are being used in an attempt to evaluate this hypothesis.

a)

	IL-1	IL-2	IL-6	IL-10	GM-CSF	β-actin	
IL-2 neg.				○	○	●	DBA/2
IL-2 pos.	●	●	●	●	●	●	mice
IL-2 neg.	○		●		●	●	nude
IL-2 pos.	●	●	●	●	●	●	mice

b)

	IL-2 Rα	IL-2	IL-4	IFN-γ	CD69	β-actin
naive	○				○	●
IL-2 neg.	○	○			○	●
IL-2 pos.	●	●	●	●	●	●

Tab. 3:
a) M–3 cells were irradiated and transfected with IL–2 plasmid. DBA/2 mice or nude mice were immunized with $5x10^5$ irradiated, or IL–2 transfected irradiated M–3 cells. Injection sites were excised at 6 h post immunization followed by purification of total mRNA.
b) Groups of DBA/2 mice (5 animals each) were immunized with irradiated, or IL–2 transfected irradiated M–3 cells. Five days after immunization draining lymph nodes were removed from immunized mice and from naive DBA/2 mice. Total RNA was prepared from $5x10^6$ pooled cells of lymph node single cell suspensions.
All RNAs were reverse transcribed using oligo dT(16) primer and MuLV Reverse Transcriptase, followed by PCR amplification with mRNA–specific sets of primers for IL–2 receptor, CD69 T cell activation marker, and different cytokines. PCR reactions were 30 sec at 95°C, 60 sec at 60°C, and 90 sec at 72°C for 40 cycles. β–actin mRNA was used to gauge efficiency of the RT–PCR in all amplifications.
● detectable after 30 PCR cycles; ○ detectable after 40 PCR cycles.

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Leukemia- and Carcinoma Formation: Mechanisms Involved in the Function and Cooperation of Oncogenes and their Normal Counterparts

Senior Scientist	Hartmut BEUG
Staff Scientist	Peter STEINLEIN
Postdoc	Marieke von LINDERN
Postdoc	Martin OFT
Postdoc	Kim C. LIM (since Oct.1994)
PhD student	Irene FIALKA
PhD student	Oliver WESSELY
PhD student	Georg MELLITZER
PhD student	Anton BAUER (since Oct. 94)
Diploma student	Martin SCHWARZ (until Sept. 94)
Technician	Evi DEINER
Technician	Liesbeth BOER
Guest	Bianca HABERMANN (March -Aug. 94)
Guest	Eugen ULRICH (April - Sept. 94)

Introduction

In 1994, we continued our work on the two main research topics of the group, leukemogenesis in the avian system, and mammary carcinogenesis in the mouse. In both areas, unexpected new findings prompted us to initiate several new directions of research.

In the avian system, we focus on the question how receptor tyrosine kinases and nuclear hormone receptors of the steroid/thyroid hormone receptor superfamily act and cooperate in the regulation of normal hematopoiesis. In addition to gaining a better understanding of normal hematopoietic differentiation, a major long-term goal of these studies is to understand how the two oncogenes of the avian erythroblastosis virus (AEV), v-*erb*A and v-*erb*B, cooperate to cause rapid, fatal erythroleukemia in young chicks. The v-ErbB protein is a heavily truncated version of the receptor tyrosine kinase TGFαR/c-ErbB, the avian homolog of the epidermal growth factor receptor that uses mammalian TGFα as a ligand. The v-ErbA protein represents a mutated version of the thyroid hormone receptor α (TRα/c-ErbA).

In 1993, we developed methods to culture two types of normal erythroid progenitors in vitro. These progenitors differ by their expression of receptor tyrosine kinases and by their ability to self renew. SCF progenitors resemble normal BFU-E/CFU-E, express the receptor tyrosine kinase c-Kit and are unable to undergo prolonged self renewal. In contrast, 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 (Hayman *et al.*, 1993). Overexpression of exogenous c-Kit and c-ErbB showed that this difference in the ability of c-ErbB and c-Kit to induce sustained self renewal was most likely due to activation of different intracellular signaling pathways by the two receptors (Wessely *et al.*, in preparation). Accordingly, we have started to analyze different signaling pathways potentially activated by these receptors (Georg Mellitzer, Oliver Wessely, together with M. Hayman, Stonybrook).

Secondly, a more detailed analysis of normal SCF progenitors revealed that they exhibit an altered behavior with respect to cell size control during the cell cycle when induced to differentiate. Proliferating cells including the self-renewing SCF/TGFα progenitors are able to "measure" their cell size at the so-called restriction point prior to entering S phase. The fact that this fundamental control point in the cell cycle might be abolished or altered after differentiation induction prompted us to initiate an investigation of the cell cycle in self-renewing and differentiating erythroid progenitors at the molecular level. (H. Beug, together with H. Dolznig and E. Müllner, Vienna Biocenter, University of Vienna).

A third "new" direction of research originated from analyzing the function of the estrogen receptor in normal erythroid progenitors and its possible functional replacement by the second AEV oncogene, v-e*rb*A.

We found that normal erythroid progenitors expressing v-ErbA no longer required an active estrogen receptor for continuous self renewal, instead, the receptor tyrosine kinases c-ErbB and c-Kit were both able to cooperate with the v-ErbA protein in self renewal induction. This finding prompted us to concentrate again on the v-ErbA oncoprotein, in particular on the mechanism how it cooperates with endogenous receptor tyrosine kinases of normal erythroblasts [Anton Bauer, Peter Steinlein, Marieke von Lindern and Hartmut Beug, in cooperation with Henk Stunnenberg (EMBL, Heidelberg) and Bjorn Vennström (Stockholm)].

In our ongoing trials to analyze the function of growth factor receptors and transcription factors in the regulation of normal erythropoiesis and leukemogenesis (see Report 1993), the focus in 1994 was entirely on analyzing in chicken cells the function and cooperation of altered gene products that together cause the Friend disease in mice. In this erythroleukemia induced by the Friend erythroleukemia virus complex, three alterations contributing to the leukemia are known: a viral glycoprotein constitutively activating the erythropoietin (Epo) receptor, constitutive expression of the transcription factor Spi-1 (Pu-1) and loss or mutation of the tumor suppressor gene p53. We have analyzed the function of each of these genes in erythroid progenitors and taken first steps to analyze their cooperation.

Finally, in 1994 we concentrated on searching oncogene combinations and growth conditions that would induce the self renewal of multipotent chicken hematopoietic progenitors which retain the ability to differentiate into several hematopoietic lineages. Firstly, multipotent progenitors transformed by the v-Gag-Myb-Ets fusion protein of ts 21-E26, a temperature-sensitive mutant of the avian myeloblastosis virus E26 (Graf *et al.*, 1992) were used to study whether the estrogen receptor and v-ErbA would have a function in lineage commitment (Marieke von Lindern). And secondly, the nuclear oncogene v-ski in cooperation with a ligand-activated c-Kit receptor tyrosine kinase (Larsen *et al.*, 1993) caused the prolonged self renewal of avian multipotent progenitors, able to differentiate into erythrocytes, mast cells, macrophages and probably neutrophils upon removal of SCF and addition of suitable cytokines (H. Beug and O. Wessely, with M. Hayman, Stonybrook, New York).

The second major interest of the laboratory is carcinogenesis. In this process, multiple genetic events including the activation of dominant oncogenes as well as loss or mutation of tumor suppressor genes lead to the transformation of epithelial cells. Our group focuses on the analysis of how single oncogenes involved in signal transduction (e.g. growth factor receptors, intracellular signaling proteins and transcription factors activated by intracellular signaling) would affect key features of epithelial cells. These are: epithelial polarity, i.e. the ability of epithelial cells to direct different proteins to the apical or the basolateral face of the epithelial cell (Simons and Fuller, 1985), and epithelial-mesenchymal transitions, i.e the regulated loss of epithelial polarity occurring during embryogenesis or tissue remodeling (Hay, 1990). We employ mammary epithelial cell lines of the mouse that fully retain the ability to build up normal epithelial polarity in culture. In 1994, we have finished our studies on a conditionally activated c-Jun-steroid receptor fusion protein (c-Jun-ER). The c-Jun protein, a constituent of the AP-1 transcription factor, is at the end of a protein kinase signaling cascade activated by growth factor receptors. The second major project in 1994 was to elucidate how the Ha-Ras oncoprotein (a constitutively active version of the major signal transduction protein c-Ras involved in receptor tyrosine kinase signaling) induces epithelial-mesenchymal transition in mammary epithelial cells and how this event relates to tumor formation. (Martin Oft, in collaboration with Lisa Ballou and W. Birchmeier, Berlin).

A. Normal erythropoiesis and leukemogenesis

1. Normal erythroid progenitors: Regulation of self renewal and differentiation by receptor tyrosine kinases

Self renewal is defined as the ability of progenitor cells to proliferate without entering a differentiation pathway. In normal hematopoiesis, self renewal is thought to be restricted to the pluripotent stem cell, but cells with the properties of committed progenitors were shown to self renew in many leukemias. In 1993, we challenged the notion that only pluripotent stem cells self renew by describing normal, committed erythroid progenitors capable of sustained self renewal. These cells (SCF/TGFα progenitors) coexpress the c-Kit receptor tyrosine kinase and the avian epidermal growth factor receptor (c-ErbB) and undergo continuous self renewal in response to the c-ErbB-ligand [transforming growth factor (TGF) α] and estradiol (Schroeder *et al.*, 1993). The common erythroid progenitors, termed SCF progenitors or BFU-E / CFU/E, express c-Kit but only undergo a limited number of cell divisions in response to the c-Kit ligand, stem cell factor (SCF). Both progenitors faithfully reproduce terminal erythroid differentiation in vitro when exposed to differentiation factors. In 1994, we have concentrated on trying to gain first insights into how self-renewing and differentiating erythroid progenitors might differ at the molecular level, how self-renewing erythroid progenitors develop during hematopoiesis and by which mechanisms c-ErbB would cause self renewal of committed erythroid progenitors.

Molecular events during normal erythroid progenitor differentiation

(P. Bartunek, M. Zenke and H. Beug).

In 1993, we showed that mass cultures of SCF- or SCF/TGFα progenitors yielding cell numbers large enough for extensive biochemical analysis could be induced to synchronously differentiate into mature erythrocytes by the same types of differentiation factors (Epo-containing anemic chicken serum and insulin) as required by normal human erythroid progenitors. The differentiation induced cells underwent five cell divisions during the first 3 days of differentiation and then arrested in G1. The final steps to fully mature erythrocytes occurred in the absence of further cell divisions. In the first 16 to 24 hours after differentiation induction, the gene expression pattern of these cells was extensively reprogrammed. Genes characteristic of self renewing cells (c-*myb*, c-*kit*, estrogen receptor) abruptly ceased to be expressed whereas erythroid-specific transcription factors such as GATA-1, GATA-2, SCL and NF-E2 were strongly upregulated, followed slightly later by activation of numerous late erythrocyte genes (see Report 1993). These results suggest that erythroid differentiation may be turned on by "master switch" regulators, the molecular nature of which is so far unknown.

Cell cycle behavior and size control during differentiation

(H. Beug, with H. Dolznig and E. Müllner, Vienna Biocenter, and K. Nasmyth, IMP).

When induced to differentiate, normal erythroid progenitors proliferate much faster than cells from the same preparation maintained in self renewal factors. At the same time, the cells progressively reduced their size with each division. We therefore asked whether erythroid differentiation may be accompanied by changes in cell cycle regulation including an altered or absent size control (somatic cells have to grow to twice the size after mitosis before they can enter another round of mitosis).

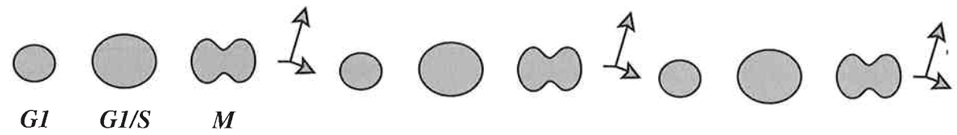
Closer investigation of differentiating and self renewing SCF and SCF/TGFα-progenitors revealed the following: After differentiation induction, cells continued to grow with cell cycle times of 17-20 hours for 12 to 16 hours (i.e. for the length of roughly one cell cycle). Thereafter, cell cycle times were reduced to 11-12 hours and stayed that short for the remaining 4 cell divisions, until 60-70 hours after differentiation induction. Thereafter, the cells rapidly withdrew from cycle. Measurements of cell size, using a new, computerized cell counter allowing exact size measurements (CASY-I, Schärfe-System) revealed that the maturing cells reduced their diameter from ≈ 8 μm to 5.1 μm, corresponding to a volume change from 350 femtoliters (fl) to 80-90 fl. This reduction in cell size started 12 to 16 hours after differentiation induction and proceeded until the cells became postmitotic (72 hours). No such size changes (SCF/TGFα-progenitors) or much less drastic ones (SCF progenitors) were observed in self-renewing cells. The changes in cell size could be confirmed by protein determinations. When the total protein synthesis rate was measured, no increase in the speed of protein synthesis was observed that could account for the shortened cell cycle. Rather, protein synthesis rate normalized to cell volume remained essentially constant.

These results suggested that differentiating SCF progenitors may indeed exhibit a cell cycle with altered size control. It was therefore of interest to determine cell cycle parameters, such as the duration of the G1, S and G2 phases. The results clearly show that shortening of the cell cycle from 17-20 hours to 11-12 hours is essentially due to a respective shortening of the G1 phase (from 10-11 hours to 4.5 - 5.5 hours). G2 is also slightly shortened (2.5 to 2 hours) while the S phase is not affected (5.5 to 6 hours). Since correct cell size seems to be checked at the G1-S transition and is a prerequisite for entry into S (Pardee, 1989), our observation that the differentiating SCF progenitors exhibit a drastically shortened G1 phase is in accord with our assumption of an altered size control in these cells (see **Fig. 1**).

To analyze the difference between "self renewal divisions" and "differentiation divisions" (see **Fig. 1**) at the molecular level, several prerequisites have to be fulfilled. Firstly, both self renewing and differentiating normal erythroid progenitors have to be separated into fractions containing cells at only one phase of the cell cycle. This problem has been overcome by developing techniques to separate large scale preparations of these cells (500-800x10⁶) by centrifugal elutriation. Pure fractions of viable cells in G1, late G1, S, early and late G2 and in mitosis were obtained, containing enough cells (20-50x10⁶) for biochemical analyses. Methods to recultivate these fractions after elutriation to avoid artefacts induced by the elutriation process are currently developed.

Secondly, 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 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 has been developed. This method involves a comparison between the mammalian and *Xenopus* genes and designing appropriate conserved PCR primers. Trials to clone the missing components by this method are currently underway.

Self-renewal **divisions**



- Cell cycle time: 19 hours, G1: 10 hours
- Cell size at G1/S constant

Differentiation **divisions**

- Cell cycle time: 12 hours, G1: 5 hours
- Cell size at G1/S progressively decreasing

Fig. 1: *Terminal erythroid differentiation may involve altered cell cycle control.* Our present view of how cell cycle control is altered during terminal erythroid differentiation is shown. In *self renewal* divisions, (top panel) both the cell cycle and the G1 phase are of standard durations and the cells maintain a constant size at the G1/S transition. In contrast, *differentiation* divisions comprise a shortened cell cycle, in which the length of the G1 phase is drastically reduced while S and G2/M remain constant. At the same time, cell volume progressively drops from about 300 to 70 femtoliters during these five cell divisions.

Developmental origin of SCF- and SCF/TGFα-progenitors

(H. Beug and P. Steinlein, with M.Hayman, New York).

Our demonstration that normal avian progenitors committed to the erythroid lineage are capable of sustained self renewal under specific conditions raised the question how these progenitors were generated during normal hematopoiesis. In 1993, we demonstrated that the simplest hypothesis, i.e. that SCF/TGFα-progenitors corresponded to BFU-E (burst forming unit erythroid) and developed into CFU-E (colony forming units erythroid; corresponding to SCF progenitors) by undergoing several cell divisions was not correct. Rather, our data suggested that both progenitors are generated through parallel, but independent pathways or that SCF/TGFα progenitors develop from SCF progenitors, acquiring self renewal potential only in the presence of certain specific combinations of growth factors and hormones.

In 1994, we were able to generate data that clearly favored the second alternative. (**Fig. 2A**). When normal SCF progenitors are cultivated in a combination of SCF, TGFα, estradiol and tested batches of chicken serum, a large proportion of these cells no longer cease to proliferate and undergo apoptosis as seen when grown in SCF alone. Instead, the cells start to express increasing levels of TGFαR/c-*ErbB* and gradually acquire sustained self renewal capacity. After 10-14 days, the cells were able to proliferate in the presence of TGFα and estradiol only, suggesting they had converted into SCF/TGFα progenitors.

These findings obtained in mass cultures could still be interpreted by the assumption that cells expressing c-ErbB were present in the SCF progenitors at low level and overgrew the SCF progenitors. To rule this out and directly demonstrate on the single cell level that non-self renewing SCF progenitors can develop into SCF/TGFα progenitors capable of continuous self renewal, an approach involving limiting dilution cloning of SCF progenitors and following the fate of individual clones in the presence of various factor combinations was used. We reasoned that in the case of the first possibility (overgrowth by rare, preexisting SCF/TGFα progenitors) most clones growing in SCF, TGFα, estradiol would cease to grow after 8-10 days, leaving only few surviving clones corresponding to the preexisting SCF/TGFα progenitors. Alternatively, most clones present after 4-6 days should persist in presence of all three factors, if SCF progenitors did indeed develop into SCF/TGFα progenitors under these conditions.

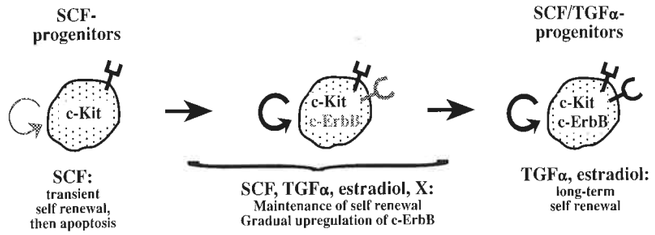
The results clearly showed that the second alternative was true. Limiting dilution cloning of both purified SCF progenitors and total, unfractionated bone marrow revealed that > 50% of the colonies developing in SCF, TGFα and estradiol survived under the latter conditions but died after 6-10 days in SCF alone. Many of the clones developing in SCF, TGFα and estradiol could be expanded into mass cultures and shown to express c-ErbB and to proliferate in TGFα and estradiol only.

In a final series of experiments, we showed by the use of chicken sera depleted for endogenous growth factors and hormones that SCF/TGFα progenitors do not develop from SCF progenitors, if any one of the three factors (SCF, TGFα or estradiol) is absent. By the same approach, we also demonstrated that the generation of the self renewing progenitors in presence of SCF, TGFα and estradiol is strongly enhanced by an unknown growth factor activity present in chicken serum. Attempts are currently underway to determine whether this activity is a growth factor or a steroid hormone and to clarify its molecular nature.

2. Differential induction of self renewal by the receptor tyrosine kinases c-Kit and c-ErbB: Identification of possible differences in intracellular signaling

The finding that SCF progenitors expressing c-Kit only were unable to continuously self renew while c-ErbB/c-Kit expressing SCF/TGFα progenitors were able to do so raised the question whether c-Kit and c-ErbB could transduce the same signal eventually leading to self renewal, but expression levels of the two receptors and/or regulation of such levels during self renewal and differentiation would be sufficiently different to explain the different behavior of the two cell types. Alternatively, the signal transduction pathways used by c-Kit and c-ErbB could be different, thus rendering c-ErbB but not c-Kit capable of inducing long-term self renewal. In 1993, we demonstrated by overexpressing c-Kit and c-ErbB in suitable cells that the second alternative was most likely true. In 1994, we have used two different approaches to elucidate whether c-Kit and c-ErbB show qualitative differences in the signal transduction pathways they activate. The first approach consisted of specifically inhibiting the biological function of c-ErbB or c-Kit, using different types of low molecular weight tyrosine kinase inhibitors as well as neutralizing antibodies. In a second approach, we made use of commercially available antibodies to identify components of known signaling pathways, that may be preferably activated by c-ErbB on one hand or by c-Kit on the other.

A. Development of self-renewing erythroid progenitors



B. Specific signaling pathways involved in c-ErbB-driven self renewal ?

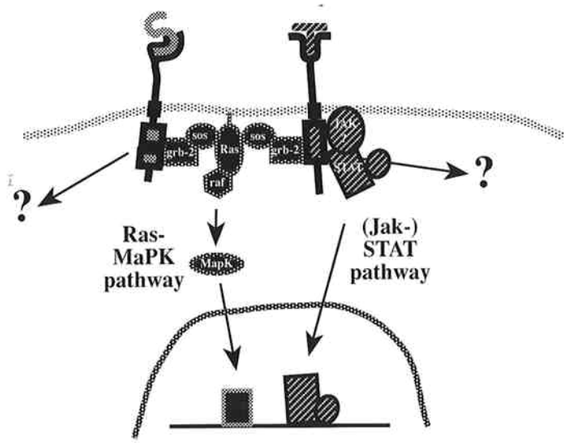


Fig. 2:

A. *SCF/TGFα progenitors develop from SCF-progenitors.*
This simple scheme depicts our current hypothesis that self renewing SCF/TGFα progenitors (thick circular arrow) develop from SCF progenitors capable only of transient self-renewal (thin circular arrow) when exposed to a combination of SCF, TGFα, estradiol and an unknown factor X present in chicken serum. During development of these progenitors which requires several cell divisions (large horizontal bracket), c-ErbB (circular receptor symbol) is gradually upregulated from low (grey receptor symbol) to high levels (black receptor symbol. The main function of SCF-activated c-Kit (rectangular receptor symbol) in this process may be to suppress apoptosis until the cells express c-ErbB levels sufficient to maintain self renewal.

B. *c-ErbB specific signaling pathways involved in self renewal induction ?*
This cartoon illustrates our current, speculative view how the differential action of the receptor tyrosine kinases c-Kit (receptor symbol with ligand S) and c-ErbB/TGFαR (receptor symbol with ligand T) on erythroblast self renewal may be explained. It is possible that c-ErbB but not c-Kit activate the JAK/STAT pathway (hatched protein symbols) while both receptors activate the Ras-MapK pathway. In addition, both receptors may activate additional specific signal transduction pathways (arrows with question marks).

Dissection of the signal transduction pathways of c-Kit and c-ErbB by tyrosine kinase inhibitors

(O. Wessely, M. von Lindern and H. Beug, in cooperation with T. von Rüden and F. Himmelsbach, Boehringer Ingelheim).

In 1993, we reported the use of a group of tyrosine kinase inhibitors referred to as "tyrphostins" in trials to specifically inhibit c-Kit and c-ErbB. These compounds are thought to block the activation of downstream signaling mediated by the interaction of autophosphorylated receptor with SH2 domain containing proteins. Out of 15 compounds tested, one turned out to be relatively specific for c-ErbB, one was specific for c-Kit and one blocked the activation of both. However, the specificity of these compounds was limited and they had to be applied in rather high concentrations. Recently, we obtained much cleaner results with another type of inhibitors (kindly provided by Dr. F. Himmelsbach, Thomae, Biberach), thought to specifically bind to the ATP-binding domain of the c-ErbB kinase. Using these inhibitors we could show that they totally abolished self renewal induction by ligand activated c-ErbB in SCF/TGFα progenitors, while they did not affect the proliferation of SCF progenitors. In line with this, the inhibitors completely blocked the outgrowth of self-renewing erythroid progenitors in TGFα plus estradiol while the outgrowth of the transiently self-renewing SCF progenitors was not affected. Finally, these inhibitors completely blocked the autophosphorylation of c-ErbB after stimulation with TGFα in Western blot analysis using anti-phosphotyrosine antibodies. In the same assays, no effect on autophosphorylation of c-Kit induced by SCF was seen. Also, the inhibitors complete-

ly failed to affect the function of the Epo receptor or the insulin receptor function during erythroid differentiation, since SCF/TGFα progenitors cultivated in TGFα as well as Epo and insulin underwent normal differentiation into mature erythrocytes in the presence of these inhibitors, while the same cells continued to self renew in absence of the inhibitors. These results strongly strengthened our notion that c-ErbB and c-Kit activate different signal transduction pathways.

Specific signaling pathways activated by c-ErbB and c-Kit ?

(G. Mellitzer and O. Wessely, in cooperation with M. Hayman, Stonybrook).

In a second approach, we tried to identify components of known signaling pathways that may be preferably activated by c-ErbB on one hand or by c-Kit on the other. Since the Ras/MAPK-signaling pathway was known to be activated by both receptors in a similar fashion, we decided to look at components of a Ras-independent signaling pathway. Receptor activation by ligand leads to tyrosine phosphorylation of p91/84 STAT1α/β, thereby causing the translocation of these **S**ignal **T**ransduction **A**ctivated **T**ranscription factors to the nucleus, where they activate specific genes. (**Fig. 2B**). Analysis of TGFα/SCF dependent progenitors showed a dramatic difference in the tyrosine phosphorylation of these transcription factors depending on whether c-ErbB or c-Kit were ligand-activated in these cells. Both STAT-1α and STAT-1β became tyrosine-phosphorylated after activation of the c-ErbB receptor, while c-Kit seemed to cause only the phosphorylation of STAT1β but not of STAT1α. In line with this, inactivation of c-ErbB function by tyrosine kinase inhibitors (see preceding paragraph) abolished any phosphorylation of STAT1 proteins, while phosphorylation of STAT1β by ligand-activated c-Kit was not affected.

These results constitute the first tentative evidence that signaling pathways activated by c-Kit and c-ErbB may indeed be different (**Fig. 2B**). Since phosphorylated STAT1α functions as a transactivator while phosphorylated STAT1β seems only to act as a repressor of transcription, the above results may already open a way to study how the qualitative differences postulated to exist between c-ErbB- and c-Kit- induced signaling may lead to different cell phenotypes.

3. Normal and mutated steroid/thyroid hormone receptors: Mechanisms of action in normal erythropoiesis and leukemia

As mentioned above, both the induction of self renewal in normal erythroid progenitors and the induction of erythroleukemia require the cooperation of the normal or mutated receptor tyrosine kinases (c-Kit, c-ErbB, v-ErbB) with members of the steroid/thyroid hormone receptor superfamily (TR-α/c-ErbA, ER, v-ErbA). Here we will briefly discuss the progress made in understanding how the estrogen receptor (ER) and the thyroid hormone receptor (TR-α/c-ErbA) regulate self renewal and differentiation in erythroid cells and then present some new results suggesting that the mutated thyroid hormone receptor α, v-ErbA, not only antagonizes TR-α/c-ErbA and functionally replaces the ER, but also intimately cooperates with c-Kit to induce prolonged self renewal in the infected progenitors.

The protooncogene TR-α/c-ErbA induces erythroid differentiation

(M. von Lindern, in collaboration with H. Stunnenberg, Heidelberg)

SCF progenitors and SCF/TGFα progenitors express both the protooncogene TRα-1/c-ErbA and the closely related retinoic acid receptors (RAR) α and γ, although expression levels may vary (Gandrillon *et al.*, 1994). Upon activation by ligand (thyroid hormone, T_3 or retinoic acid, RA), these receptors were shown to induce a complex response in the self-renewing, normal erythroid progenitors. When transiently ligand-activated for 36 to 48 hours, TR-α/c-ErbA and RARα triggered complete, Epo plus insulin-dependent erythroid differentiation despite the presence of self-renewal factors (**Fig. 3A**). If applied throughout differentiation (or added to partially mature cells), T_3 or RA caused only a transient maturation, but then inhibited further cell proliferation and caused cell death by apoptosis. However, recent experiments using media stripped of endogenous RAR-ligands and using T_3 in combination with ligands specific for RXR, the heterodimerization partner of TR-α/c-ErbA and RAR, suggested that this apoptosis induction requires participation of ligand-activated RAR.

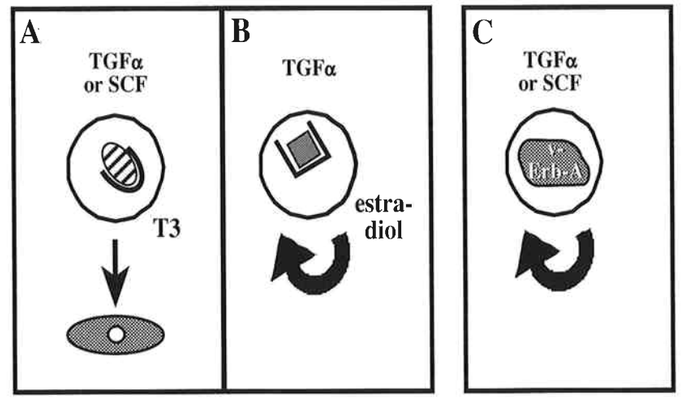


Fig. 3: *Antagonistic effect in erythroid progenitors of the nuclear hormone receptors for thyroid hormone and estradiol and the role of the mutated thyroid hormone receptor v-ErbA.*
This scheme indicates that the ligand-activated thyroid hormone receptor (panel A, elliptical receptor-ligand symbol) induces erythroid differentiation in both SCF- and SCF/TGFα progenitors exposed to SCF or TGFα, while the ligand-activated estrogen receptor (ER; panel B, rectangular receptor-ligand symbol) enhances self renewal induced by TGFα in SCF/TGFα progenitors. V-ErbA enhances self renewal induction in SCF/TGFα progenitors exposed to TGFα even if the endogenous ER is inactivated by an antagonist (panel C, note absence of the ER symbol) and induces SCF progenitors to prolonged self renewal (circular arrow) in presence of SCF only. This supports our current view that v-ErbA may not only be a dominant negative version of c-ErbA, but also behave like a constitutively active ER.

Estrogen receptor function in erythroblast self renewal: Repression of erythrocyte genes does not require binding to specific DNA elements

(M. von Lindern, in collaboration with M. Parker, London).

In 1993, we showed that the estrogen receptor inhibited or retarded erythroid differentiation and arrested expression of several erythroid-specific genes at the transcriptional level (**Fig. 3B**). This inhibition functioned in partially mature erythroid cells, suggesting that the ER not only prevents entry into cell differentiation, but can block differentiation also at later stages. It was unclear, however, whether repression of erythroid genes by the ER was a direct effect, since none of the repressed genes were turned off rapidly (i.e. within 1-2 hours). In trials to circumvent these difficulties, we asked which domains of the estrogen receptor were important for repression of erythroid differentiation and altered regulation of erythrocyte-specific genes. For this, mutant human and mouse receptors carrying deletions or inactivating point mutations in the DNA binding domain, the C-terminal transactivation domain AF-2 and the N-terminal transactivation domain AF-1 were inserted into retroviral vectors and used to infect an erythroblast line devoid of endogenous ER (HD-3) as well as primary erythroblasts. To our surprise, ER mutants unable to bind to an ERE or even to DNA still blocked erythroid differentiation and repressed erythrocyte specific genes. In the presence of estradiol, the more severe DNA binding mutant even seemed to be toxic to the cells. Deletion of the AF-1 domain enhanced differentiation arrest and gene repression by the ER, while mutation of the AF-2 domain rendered the ER biologically inactive. These results suggest that the ER is not required to bind to an ERE in order to repress genes. Rather it could function by interaction with other transcription factors or components of the basic transcriptional machinery. Interestingly, this situation was not true for the CAII gene, which was shown to be upregulated by the ER (see Report 1993). Here, mutants of the ER deficient for DNA-binding failed to upregulate the CAII gene, suggesting that its regulation by the ER required specific DNA binding.

The v-ErbA oncoprotein functionally replaces the estradiol receptor in causing erythroid progenitor self renewal and differentiation arrest

(E. Ulrich, M. von Lindern and A. Bauer).

The functional analogy between the ER and v-ErbA (both caused an arrest of erythroid differentiation and inhibition of erythroid-specific genes) further prompted us to investigate whether v-ErbA could fulfill another function of the ER, i.e. to sustain self-renewal of primary erythroblasts. For this, normal erythroid progenitors were infected with a retrovirus expressing v-ErbA and cultivated in the presence of receptor tyrosine kinase ligands (SCF or TGFα) but in the absence of a ligand-activated ER (i.e. in presence of the ER antagonist ICI 164384). To our surprise, v-ErbA was indeed able to cause sustained self renewal of erythroid cells in the presence of either SCF or TGFα in presence of the ER antagonist, while control cultures infected with an empty vector completely failed to self renew under these conditions. In addition, clear differences were seen between the constitutive actions of v-ErbA and the ligand activated function of the ER. Estradiol-dependent, long-term erythroid self-renewal occurs only in erythroid precursors that express both TGFαR/c-ErbB and cKit, the receptor for SCF. V-ErbA, on the other hand, promoted sustained self renewal also in erythroid precursors that lack c-ErbB and express c-Kit only (**Fig. 3C**). In the absence of v-ErbA, these progenitors are unable to undergo sustained self renewal under any culture conditions known so far.

Trials to demonstrate that v-ErbA would also arrest differentiation in normal progenitors presented an enigma. Surprisingly, the v-ErbA expressing, normal progenitors were able to terminally differentiate in the presence of differentiation factors (Epo plus insulin), suggesting that v-ErbA is unable to arrest differentiation in these progenitors. In contrast, v-ErbA completely arrested differentiation when the cells were grown in a combination of SCF with Epo plus insulin, while control erythroblasts underwent a slightly delayed but complete differentiation program under the same conditions. Thus, v-ErbA seems to require the presence of a ligand-activated c-Kit receptor tyrosine kinase for an efficient arrest of erythroid differentiation and repression of gene expression while it is unable to exert these repressing functions in absence of a lig-and-activated receptor tyrosine kinase. These results strongly suggest that signal transduction activated by the receptor tyrosine kinase and leading to serine phosphorylation of v-ErbA [which has been shown earlier to be required for its repression function, (Glineur *et al.*, 1990)] is indispensable for v-ErbA function (**Fig. 4A**). These results also suggest a testable mechanism through which oncogene cooperation between v-ErbB and v-ErbA could occur (**Fig. 4B**). It is unclear at present, whether the ER also requires signaling from a receptor tyrosine kinase for repressive activity.

Finally, our analysis of the expression of proteins known to be involved in signal transduction (see above) identified a candidate molecule [**E**GF-receptor **p**rotein **s**ubstrate (Eps) 8], that may be somehow involved in this signal transduction process. Eps 8 was initially identified as a substrate of the activated EGF-receptor (Fazioli *et al.*, 1993). It was highly expressed in SCF-dependent progenitors, but decreased during their development into SCF/TGFα dependent progenitors, in which it was low or absent. Since pilot experiments suggested that Eps 8 was not downregulated in self renewing SCF-dependent progenitors expressing v-erbA and that it was even induced in SCF/TGFα dependent progenitors after forced expression of v-ErbA, it may well represent a possible candidate involved in the signaling between c-Kit and v-ErbA.

4. Transcription factors and tumor suppressors in leukemia: Model studies in primary avian hematopoietic cells.

Reconstructing murine Friend erythroleukemia in avian erythroblasts

(H. Beug and M. van Lindern, with J. Ghysdael, Paris).

In 1993, we initiated studies with the aim to determine how the single oncogenes involved in the murine Friend erythroleukemia contribute to the leukemic phenotype. The products of these oncogenes are: a viral glycoprotein constitutively activating the Epo receptor, two transcription factors of the Ets family, Spi-1/Pu-1 and Fli-1, which are aberrantly overexpressed due to retroviral insertion into the promoters of these genes and a deleted or mutated tumor suppressor gene p53. In an attempt to determine how these single oncogenes contribute to the leukemic phenotype, we have expressed the murine Epo receptor, Spi-1 (both as a constitutive and a hormone-inducible version) and a ts mutant of p53 in normal erythroid progenitors as well as in erythroblasts transformed by the temperature-sensitive and ts-v-sea-transformed progenitors.

Spi-1. Our results confirmed the preliminary finding reported in 1993 that spi-1 delayed or even arrested differentiation. When introduced into ts-v-sea transformed cells, Spi-1 clearly retards differentiation after shift to 42°C. The Spi-1 cultures consisted of cells at essentially all maturation stages including a significant proportion of immature cells (20-40%) at times when the control cultures have differentiated completely. Differentiation, however, was not completely arrested, because all cells either differentiated or underwent apoptosis after 5-6 days at 42°C. To study whether the weak effect of the mammalian spi-1 could be due to partial inactivation at 42°C (which is tolerated by chicken proteins since the body temperature of the chicken is 41.8°C), we inactivated the ts-sea protein by the drug Castanospermine (Knight *et al.*, 1988) at 39.5°C. Under these conditions, the cells expressing Spi-1 were almost completely arrested in differentiation, while control ts-v-sea erythroblasts still differentiated normally.

ts-p53. In 1993, we described an avian retrovirus expressing a thermosensitive mutant of mammalian p53. Like in mammalian cells, this virus transformed avian fibroblasts at the nonpermissive temperature (39.5°C) while it arrested the fibroblasts at G1 at the permissive temperature (34.5°C). This virus was then used to infect both SCF/TGFα progenitors and ts-v-sea transformed cells. In the ts-v-sea cells, no phenotype of ts-p53 in its mutant form was seen. After shift to 42°C, the ts-p53 expressing cells differentiated normally in most clones tested. In a minority of clones, abnormally shaped erythrocytes and disintegrated cells appeared. As expected, cell proliferation was arrested at 34.5°C.

A much more interesting ts-p53 phenotype was observed in normal SCF/TGFα progenitors. After infection and expansion at 39.5°C in the presence of self renewal factors (TGFα, SCF, estradiol) for 7 days, the ts-p53 infected cells continued to proliferate in the *absence* of TGFα and SCF for 20-30 population doublings. Control cultures kept under the same conditions (i.e in estradiol only) stopped to proliferate after a few days and disintegrated. When induced to differentiate by removal of estradiol and addition of Epo plus insulin, differentiation was clearly delayed, leading to abnormally shaped, very small reticulocyte-like cells. As expected, proliferation of the ts-p53 transformed cells was completely blocked at 34.5°C. These results, originally obtained in mass cultures, could be confirmed with two ts-p53 transformed erythroblast clones obtained in semisolid medium.

Our results raise the interesting possibility that the spi-1 and p53 oncogenes cooperate in generating the complete Friend erythroleukemia phenotype. While p53 seems to be able to relax the stringent factor requirements for inducing self renewal in normal erythroid progenitors, Spi-1 is able to cause a differentiation arrest in these cells.

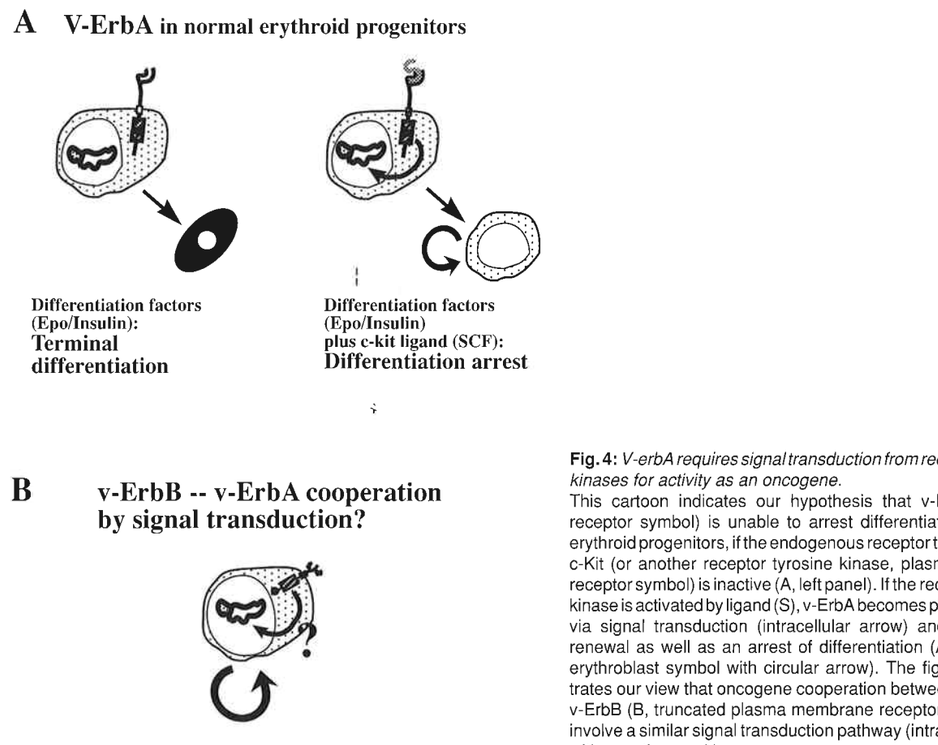


Fig. 4: *V-erbA requires signal transduction from receptor tyrosine kinases for activity as an oncogene.*
This cartoon indicates our hypothesis that v-ErbA (nuclear receptor symbol) is unable to arrest differentiation in normal erythroid progenitors, if the endogenous receptor tyrosine kinase c-Kit (or another receptor tyrosine kinase, plasma membrane receptor symbol) is inactive (A, left panel). If the receptor tyrosine kinase is activated by ligand (S), v-ErbA becomes phosphorylated via signal transduction (intracellular arrow) and causes self renewal as well as an arrest of differentiation (A, right panel; erythroblast symbol with circular arrow). The figure also illustrates our view that oncogene cooperation between v-ErbA and v-ErbB (B, truncated plasma membrane receptor symbol) may involve a similar signal transduction pathway (intracellular arrow with question mark).

Cooperation of ligand-activated EpoR with Spi-1.

Last year, we found that expression of the EpoR in normal erythroid progenitors was able to induce differentiation in the otherwise self-renewing cells. Since we found in 1994 that Spi-1 alone was unable to cause the outgrowth of normal erythroid progenitors from bone marrow and since the primary event in Friend leukemia is the constitutive activation of the EpoR by the Friend virus glycoprotein gp55, the possibility existed that signaling from the EpoR to Spi-1 was required for its function as an oncogene. We are currently constructing a retrovirus expressing both the EpoR and the Spi-1 protein to investigate this possibility.

The v-Ski oncoprotein cooperates with c-Kit to transform normal chick bone marrow cells.

(H. Beug and P. Steinlein, with R. Dahl and M. J. Hayman, New York).

Last year, we demonstrated that the v-Ski oncoprotein, a mutated transcription factor involved in muscle differentiation, could transform bone marrow cells in cooperation with the tyrosine kinase receptor c-Kit. When bone marrow cells were cocultivated with high titer v-*ski* retrovirus producing fibroblasts in the presence of avian stem cell factor (SCF), a mixture of immature or mature macrophages, neutrophils, mast-cell like cells and erythroid cells could be grown out from the infected bone marrow. These cells expressed the ski oncogene and were dependent on SCF for continuous proliferation.

Further analysis of these v-Ski-transformed cells revealed that they represented self-renewing, multipotent hematopoietic progenitors. Firstly, these cells did not senesce after 30-40 generations like any other chicken cell, but underwent 80-110 generations in culture before finally undergoing senescence, when cultivated in the presence of SCF and estradiol at high cell densities. Secondly, density fractionation of the mixed cell populations into immature and mature cell populations revealed that only one fraction of immature cells was able to proliferate in SCF plus estradiol. This fraction contained mostly immature cells which could not be clearly assigned to a specific lineage. Upon proliferation, these cells regenerated the whole spectrum of more differentiated cells, suggesting that they represented multipotent cells.

To directly demonstrate that the self-renewing v-Ski-transformed cells represent multipotent progenitors, they were cloned by limiting dilution. Cells were seeded out in 96 well plates at various densities and the number of colonies and their phenotype was determined. Besides non growing clones containing a single type of mature cell and therefore probably representing committed progenitors, numerous large colonies of either immature or immature plus mixed mature cells were obtained. These clones could be grown into mass cultures, which again contained erythroid cells, mast cells and macrophages in addition to immature cells, indicating that they represented multipotent progenitors. Further proof for this notion was obtained by the observation that several of the multipotent clones could be recloned by limiting dilution and still gave rise to multipotent subclones.

To analyze the differentiation ability of these multipotent clones, we quantitated the cell type distribution after induction of differentiation in 6 well growing clones. Anemic serum plus insulin induced myeloid and erythroid differentiation, since these sera contain cytokines supporting granulocyte/mast cell differentiation in addition to erythropoietin. The prevalence of differentiated cell types in these clones varied, both before and after differentiation induction. While some clones contained preferentially myeloid cells (macrophages and mast cells), others contained a higher proportion of erythroid cells. Experiments to further characterize the differentiation ability of these cells and trials to express foreign genes in them are currently underway.

B. Reversible loss of polarity and epithelial-mesenchymal transition: Necessary events in carcinogenesis ?

As mentioned above, the part of our group working on epithelial cells has focused on characterizing the alterations caused in polarized mammary epithelial cells by single oncogenes which are involved in the transduction of signals from cell surface growth factor receptors to nuclear transcription factors. This is of obvious relevance to human mammary carcinomas, which frequently contain amplified genes encoding the EGF receptor (HER-1) or the closely related receptor tyrosine kinase HER-2. In the mouse, a well-known model system to induce mammary carcinomas by chemical mutagenesis involves the signal transducing protooncogene Ha-Ras. We concentrate on two relevant alterations induced by such oncogenes. The first is the reversible loss of epithelial polarity induced by a fusion protein of the signal-transduction activated transcription factor c-Jun with the hormone-binding domain of the estrogen receptor (c-Jun ER). And secondly, we analyze how the activated signal transducer protein Ha-Ras causes epithelial-mesenchymal transition in vivo and in vitro, focusing on factors that cooperate with Ha-Ras in inducing this transition from a polarized epithelial to a nonpolarized, fibroblastic state.

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

(I. Leitner, in collaboration with H. Schwarz, Tübingen)

Last year we reported that c-JunER caused the breakdown of epithelial polarity, when activated by estradiol. The cells failed to build up an ordered monolayer on permeable supports, instead the cells formed unordered mono-or multilayers with wide intercellular spaces and a strongly reduced transepithelial resistance. Both apically sorted proteins (DPP IV, Muc-1) and basolaterally sorted ones (E-cadherin, β-catenin, Fodrin etc.) lost their polarized localization, i.e. they were expressed at all domains of the plasma membrane. Interestingly, all these changes were fully reversible when estradiol was removed from the cultures.

In 1994, we have characterized this system in more detail. Firstly, the behavior of the cells was analyzed in serum-free collagen gels, in which the serum was replaced by known growth factors and hormones. In absence of estradiol, the c-junER cells formed hollow tubules that consisted of fully polarized cells and resembled the structures formed by control cells (EpH4) lacking any oncogene. When estradiol was added to these cultures, the tubular structures collapsed into solid, cord-like structures within 24 hours. Immunohistochemical analysis of these structures at the light-microscopical and electron-microscopical level indicated that apically and basolaterally sorted proteins were similarly redistributed to the entire cell surface as in the cultures on permeable supports. In clear contrast to cells expressing c-FosER (see report 1992) or Ha-Ras (see below), the c-JunER expressing cells never invaded the collagen gel upon exposure to estradiol, but remained confined to the compact, cord-like structures throughout the experiment. These results clearly demonstrate that the estradiol-activated c-JunER protein causes reversible loss of epithelial polarity, but is completely unable to induce epithelial-mesenchymal transition.

We also attempted to further characterize the molecular changes accompanying this loss of epithelial polarity. As reported last year, no changes in the expression levels of junctional proteins (ZO-1, E-cadherin, β-catenin) were seen. However, the redistribution of the adherens junctional proteins E-cadherin and β-catenin involved a reduction in the strength of complex formation between these two proteins and enrichment of β-catenin in the detergent soluble fraction indicating destabilization of the interaction with the actin cytoskeleton. This notion was confirmed by immunolocalization of β-catenin at the EM-level. In the absence of estradiol, β-catenin appeared to be firmly associated with basolateral plasma membrane structures in the absence of estradiol, while it became partly cytoplasmic after activation of the c-JunER protein. Finally, we looked at tyrosine phosphorylation of β-catenin, since reversible loss of polarity induced by the oncogene ts-v-Src in another polarized epithelial cell line (MDCK) was associated with increased phosphorylation of β-catenin on tyrosine. Although easily demonstrable on v-Src expressing control cells, loss of epithelial polarity in c-JunER expressing cells was not accompanied by increased tyrosine phosphorylation of β-catenin.

Finally, we studied whether the activation of c-JunER by estradiol would alter the expression levels of other members of the AP-1 family. We also looked at the expression of proteases and their inhibitors which may be involved in alterations of cell-cell and cell-substrate adhesiveness likely to be involved in the loss of epithelial polarity. Strikingly, the expression of the c-Jun-related transcription factor JunB was heavily downregulated already after a 2 hour exposure of the c-JunER expressing cells to estradiol. Since JunB is specifically expressed in epithelia, its downregulation could well be of importance for the observed loss of

epithelial polarity. A similar, but much less prominent downregulation of JunD was also observed. Finally, expression of the c-Fos induced transcription factor Fra-1 as well as the protease UPA and the inhibitor PAI-1 stayed high in c-JunER cells plus estradiol, which are unable to build up a polarized monolayer on polarized supports, while expression of the same genes disappeared as the c-JunER cells formed a polarized monolayer in the absence of estradiol.

2. Ha-Ras induces reversible epithelial/mesenchymal transition in polarized mammary epithelial cells

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

A frequent event in human breast carcinomas is the overexpression of receptor tyrosine kinases (RTK), which signal via Ras and the MAP kinase pathway to AP-1 family members such as c-Jun and Fos, previously analyzed in our group for their effect on epithelial polarization. Last year, we therefore initiated a study on the effect of a mutated Ha-Ras oncoprotein on polarized murine mammary epithelial cells. We showed in 1993 that these cells retained a fully polarized epithelial phenotype when cultured *in vitro* on plastic or permeable supports, provided the presence of a suitable extracellular matrix. Surprisingly, they caused rapidly growing tumors when injected subcutaneously into nude mice. During tumor development, the cells could be shown to undergo epithelial-mesenchymal transition, developing into a fibroblastoid cell type that however retained the expression of certain cytokeratins. This epithelial-mesenchymal transition was accompanied by loss of epithelial markers such as E-cadherin and ZO-1, the gain of mesenchymal proteins such as vimentin or fibronectin and the upregulation of matrix proteases.

To determine in more detail under which conditions Ha-Ras expressing epithelial cells (Ep-Ras) would undergo epithelial-mesenchymal transition *in vitro*, we investigated Ep-ras cell behavior in serum-free collagen gels supplemented with either defined growth factors and hormones or with complete foetal calf serum. Under both types of conditions, normal epithelial cells form branched, tubular structures almost indistinguishable from *in vivo* glands (**Fig. 5**). When cultured under serum free conditions, Ep-ras cells also formed highly polarized, alveolar like structures and ducts. Upon addition of serum, however, these structures rapidly converted to lumen-less cords consisting of spindle-shaped, fibroblastoid cells which rapidly invaded the collagen gel. Histological analysis revealed that the structures obtained in collagen gels plus serum closely resembled the aggregates of tumor cells *in vivo*, while the epithelial ducts and alveoli obtained under serum-free conditions more resembled those of normal glands. Epithelial/mesenchymal transition in collagen gels was accompanied by the same changes in epithelial and mesenchymal markers as seen *in vivo* (see Report 1993), again including upregulation of extracellular matrix proteases like stromelysin and collagenase I.

We next analyzed if the epithelial/mesenchymal transitions induced in Ep-Ras cells in vitro or in vivo were reversible. To our surprise, the mesenchymal cells grown out from nude mouse tumors and serum containing collagen gels underwent slow reversion to a fully polarized epithelial phenotype, if just plated at very low cell numbers on plastic and allowed to form colonies. These cells again expressed E-cadherin and ZO-1, did no longer express mesenchymal markers and exhibited a high transepithelial resistance indistinguishable from polarized Ha-Ras- or uninfected control cells. Likewise, in collagen gels, the cord-like aggregates of mesenchymal-like cells obtained in the presence of serum reverted into tubular and alveolar structures consisting of fully polarized epithelial cells when serum was withdrawn.

To test if these phenotypic changes contribute to the invasive behavior of epithelial cells harboring mutated Ras, we tested the invasiveness and metastasis-forming ability in the chick embryonic heart assay (thought to be one of the most stringent in *vitro* invasiveness tests) and by intravenous injection into nude mice. In comparison to their polarized progenitors, the Ep-ras cells after epithelial-mesenchymal transition exhibited a strongly enhanced capability to invade chicken heart fragments. Likewise, in nude mice dedifferentiated Ep-ras cells caused lethal lung metastases in a time period 3-4 times shorter as observed with their still polarized progenitors. In culture, the growth rate of the two cell types was comparable. Both experiments suggested that loss of polarity and epithelial/mesenchymal transition may represent steps in the development of epithelial cells to tumor cells able to invade and metastasize.

We conclude from our results that Ha-Ras does not drastically alter the epithelial phenotype on its own. Rather, it strongly destabilizes epithelial polarity, allowing various external factors to cause loss of epithelial polarity and epithelial-mesenchymal transition. This suggests that an additional signaling pathway may cooperate with the Ras pathway and that the overexpression of receptor tyrosine kinases in human mammary carcinomas may act through hyperactivating the endogenous Ras pathway. Our ability to faithfully reproduce events in collagen gels contributing to carcinoma formation *in vivo* may greatly help to identify these factors and to elucidate this additional signal transduction pathway.

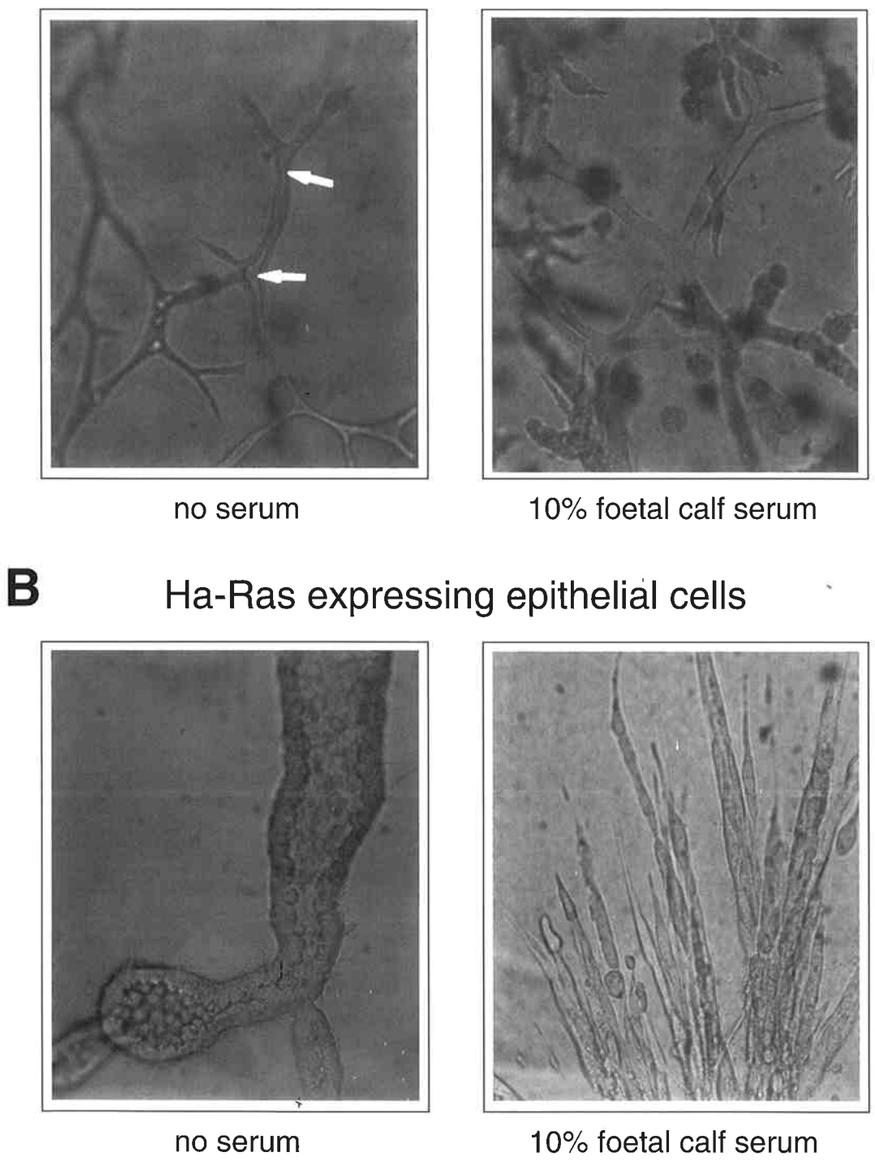


Fig. 5: *Serum induces epithelial /mesenchymal transition in epithelial cells expressing Ha-Ras.*
Photographs are shown of uninfected mammary epithelial cells (panels A) or mammary epithelial cells expressing Ha-Ras (panels B) after cultivation in serum-free collagen gels without (left panels, no serum) or with addition of foetal calf serum (right panels). While the normal cells form tubular structures (lumen indicated by white arrows) in the presence or absence of serum, the Ha-Ras expressing cells form well organized tubular structures only in the absence of serum, while they grow as unorganized clumps of cells with a spindle-like morphology, that readily invade the collagen gel.

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Transcription Factors Involved in Differentiation and Signal Transduction

Senior scientist	Meinrad BUSSLINGER
Staff scientist	Zbynek KOZMIK
Postdoc	Thomas CZERNY
Postdoc	Pavel URBÁNEK
PhD student	Petra DÖRFLER
PhD student	Arnold REIKERSTORFER
PhD student	Aline BALLENEGGER
PhD student	Susanne VAMBRIE
Technician	Paula GRANINGER

1) The role of Pax proteins in early development and differentiation

Introduction

Differential gene expression in early development and differentiation 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 paired box was initially identified as a highly conserved motif of a small gene family which is involved in segmentation and cell fate specification in *Drosophila* (reviewed by Noll, 1993). Nine members of the mammalian *Pax* gene family have since been isolated, and these genes are now known to code for transcription factors that recognize their target genes via the DNA-binding function of the paired domain (reviewed by Gruss and Walther, 1992; Stapleton *et al.*, 1993).

Like in *Drosophila*, the *Pax* genes of mammals have been implicated in the regulation of morphogenesis and pattern formation, as they are expressed in distinct, spatially and temporally restricted patterns during embryogenesis. To date, the in vivo function of three murine *Pax* genes has been studied due to their association with mouse developmental mutations. Molecular lesions in the *Pax-1*, *Pax-3* and *Pax-6* genes are responsible for the phenotypes of the *undulated*, *Splotch* and *Small eye* mice, respectively (reviewed by Gruss and Walther, 1992). The developmental abnormalities observed in these mouse mutants point to an essential role for Pax proteins in the specification, proliferation and/or migration of progenitor cells of the affected tissues. A clinically relevant aspect of *PAX* genes is their semidominance, as human disorders corresponding to the heterozygous condition of *Splotch* and *Small eye* have been identified. *PAX-3* is mutated in Waardenburg's syndrome and *PAX-6* is altered in aniridia and Peter's anomaly (reviewed by Strachan and Read, 1994).

We have previously identified the B cell-specific transcription factor BSAP as a mammalian homologue of a sea urchin transcriptional regulator (Barberis *et al.*, 1990). BSAP is expressed at early, but not late, stages of B cell differentiation and is involved in the regulation of the *CD19* gene, which codes for a B cell-specific transmembrane protein (Kozmik *et al.*, 1992). Biochemical purification and cDNA cloning revealed that BSAP is encoded by the *Pax-5* gene (Adams *et al.*, 1992). During mouse embryogenesis *Pax-5* is transiently expressed in the developing midbrain, at the midbrain-hindbrain junction and in the spinal cord, and in the adult mouse *Pax-5* expression is seen in testis and all B-lymphoid tissues (Adams *et al.*, 1992).

As reported last year, we have inactivated the *Pax-5* gene in the mouse germline in order to study the role of *Pax-5* in brain development and B cell differentiation. During the past year we have characterized the phenotype of the *Pax-5* mutant mouse in great detail. In addition, we have subjected the BSAP (Pax-5) and Pax-6 proteins to an extensive structure-function analysis to gain insight into the mechanism by which transcription factors of the Pax family regulate gene expression.

Transactivation properties of BSAP (Pax-5)

Petra Dörfler

Although we discovered the **B** cell-**s**pecific **a**ctivator **p**rotein BSAP over four years ago (Barberis *et al.*, 1990), we still know little about potential B cell-specific target genes for this transcription factor. To date, the best characterized gene is *CD19* which codes for a B cell-specific transmembrane protein involved in signal transduction. A high affinity BSAP-binding site was found in the -30 region of this gene upstream of a cluster of heterogeneous transcription start sites and was shown to be fully occupied by BSAP *in vivo* in B-lymphoid cells (Kozmik *et al.*, 1992). However, attempts to demonstrate transcriptional activity of the TATA-less *CD19* promoter in transient transfection experiments largely failed so far. Similarly, BSAP-dependent transcription could also not be convincingly demonstrated for three other putative target genes, *blk*, *Iε* and *VpreB*.

In the absence of naturally occurring promoters, which are BSAP-dependent in transfection experiments, we have created an artifical promoter by inserting multimerized BSAP-binding sites upstream of the TATA box of a rabbit β-globin gene with the aim of studying the transactivation properties of BSAP. This reporter construct was transiently transfected into plasma cell lines together with expression vectors directing the synthesis of full-length or truncated BSAP peptides. While full-length BSAP strongly stimulated transcription of this reporter gene, deletion of sequences in the C-terminal serine-, threonine-, proline-rich region of BSAP prevented transcriptional activation. Mutation of this region by N- and C-terminal resections identified a stretch of 53 amino acids as the core element of the transactivation domain (**Fig. 1**). This core element is flanked on the C-terminal side by a tyrosine-rich sequence motif which exerts a strong negative influence on the transactivation function. Hence, we have identified positive and negative elements which together determine the transactivation potential of BSAP.

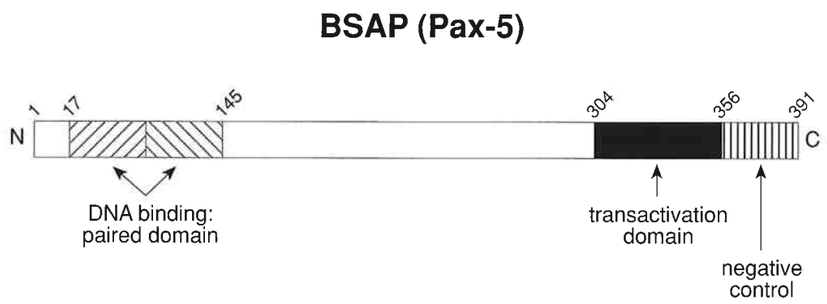

Fig. 1: Structure of the transcription factor BSAP (Pax-5).

Three amino acids in the paired domain are responsible for the different sequence specificity of BSAP (Pax-5) and Pax-6.

Thomas Czerny

Pax-6 is known to be a key regulator of vertebrate eye development. We have now isolated cDNA for an invertebrate Pax-6 protein from sea urchin embryos. Transcripts of this gene first appear during development at the gastrula stage and are later expessed at high levels in the tube foot of the adult sea urchin. The sea urchin Pax-6 protein is highly homologous throughout the whole protein to its vertebrate counterparts with the paired domain and homeodomain being virtually identical. Consequently, we find that the DNA-binding and transactivation properties of the sea urchin and mouse Pax-6 proteins are very similar, if not identical. By in vitro mutagenesis and transient transfection experiments we could localize a potent transactivation function within the C-terminal sequences of both the sea urchin and mouse Pax-6 proteins. Although both the C-terminal transactivation domains of Pax-6 and BSAP (Pax-5) are rich in serine, threonine and proline, they differ completely in primary sequence, suggesting that members of different Pax subfamilies use distinct co-activator proteins to stimulate transcription.

The consensus recognition sequence of the Pax-6 paired domain (Epstein *et al.*, 1994) is almost identical to that of BSAP/Pax-5 (Czerny *et al.*, 1993). However, the affinity for individual, naturally occurring binding sites differs largely for the two proteins. By creating chimaeric fusion proteins between Pax-6 and BSAP, we were able to demonstrate that (i) the C-terminal halves of the paired domains of Pax-6 and BSAP are functionally equivalent and that (ii) a short amino acid stretch in the N-terminal region of the paired domain is responsible for the differential binding specificity of Pax-6 and BSAP (**Fig. 2**). Mutation of three Pax-6-specific residues in this region to the corresponding amino acids of BSAP resulted in a complete switch of the DNA-binding specificity from Pax-6 to BSAP (**Fig. 2**).

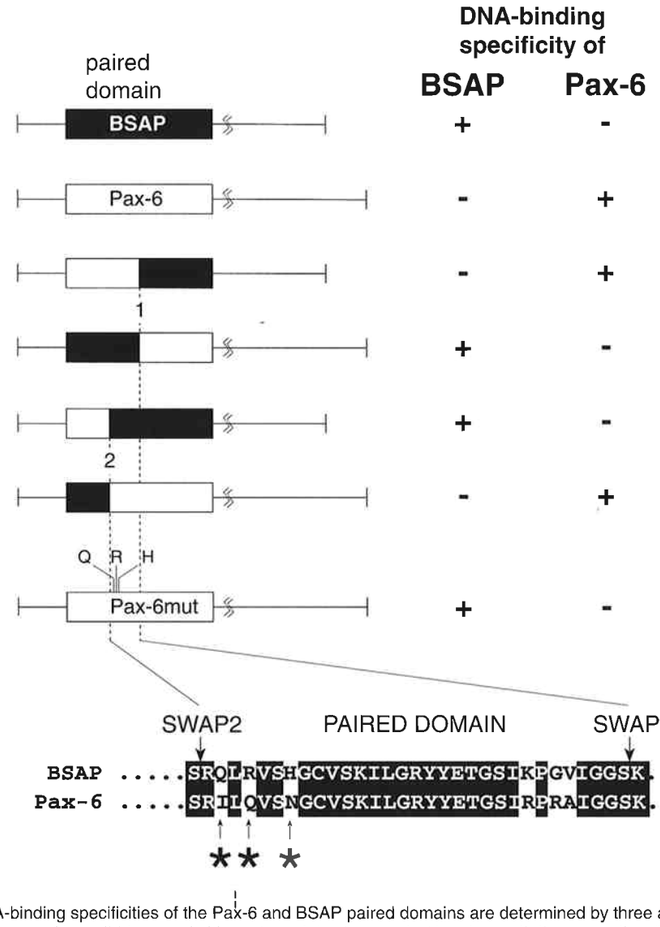

Fig. 2: The DNA-binding specificities of the Pax-6 and BSAP paired domains are determined by three amino acid residues.
A schematic diagram of the wild-type and chimaeric proteins is indicated together with the respective DNA-binding specificity. For details see Czerny and Busslinger (1995).

Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax-5

Pavel Urbánek in collaboration with Zhao-Qi Wang, Ingrid Fetka and Erwin F. Wagner

The expression pattern of the *Pax-5* gene predicts a role for this transcription factor in midbrain development, B cell differentiation and spermatogenesis. To test this hypothesis, we have generated mice carrying a germline deletion of the *Pax-5* paired box. Homozygous mutant mice were born alive, became growth-retarded and usually died within three weeks. About 5% of mutants survived to adulthood and were fertile, but severely runted. Morphogenesis of the posterior midbrain was affected as early as embryonic day 16.5 leading to a dramatic reduction of the inferior colliculus near the midline and to altered foliation of the anterior half of the cerebellum (**Fig. 3**). Moreover, all mutants failed to produce small pre-B, B and plasma

cells due to a complete arrest of B cell development at an early precursor stage (**Fig. 4**). Interestingly, this differentiation block was accompanied by the loss of expression of the Pax-5 target gene *CD19*. These data define a key role for Pax-5 in the regulation of early B-lymphopoiesis and in pattern formation of the posterior midbrain (Urbánek *et al.*, 1994).

In collaboration with Antonius Rolink at the Basel Institute of Immunology we have recently started to characterize the residual B-lymphoid cells of homozygous mutant mice in detail. These analyses revealed that the majority of the Pax-5-deficient B-lymphocytes are proliferating and large in size, express c-kit, one of the earliest markers of the B-lymphoid lineage, and have only undergone D_HJ_H rearrangement of the immunoglobulin heavy-chain gene. Similarly to the Pax-5 mutation, B cell development is also blocked at an early stage by targeted mutation of other B-lymphoid genes (*RAG-1*, *RAG-2*, *λ5* and *μMT*), which are involved in the expression of the pre-B cell receptor complex. Comparison of the cell surface phenotype and status of immunoglobulin gene rearrangement indicated, however, that B cell differentiation is arrested in *Pax-5* mutant mice at an earlier stage and by a different mechanism than in these B cell-deficient mice.

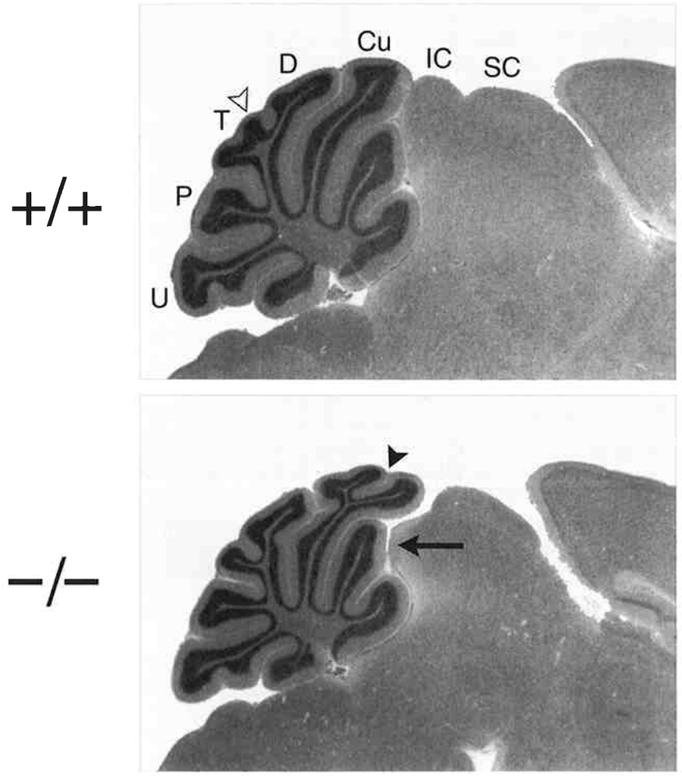

Fig. 3: Abnormal development of the inferior colliculus and the anterior cerebellum in Pax-5-deficient mice.
Midsagittal sections through the midbrain and cerebellum of 3-week-old mice are shown. The remnant of the inferior colliculus in the homozygous mutant (-/-) mouse is denoted by an arrow, and the deep intraculminate fissure present in the mutant cerebellum is indicated by an arrowhead. Abbreviations: SC, superior colliculus; IC, inferior colliculus; Cu, culmen; D, declive; T, tuber vermis; P, pyramis; U, uvula. For details see Urbánek *et al.* (1994).

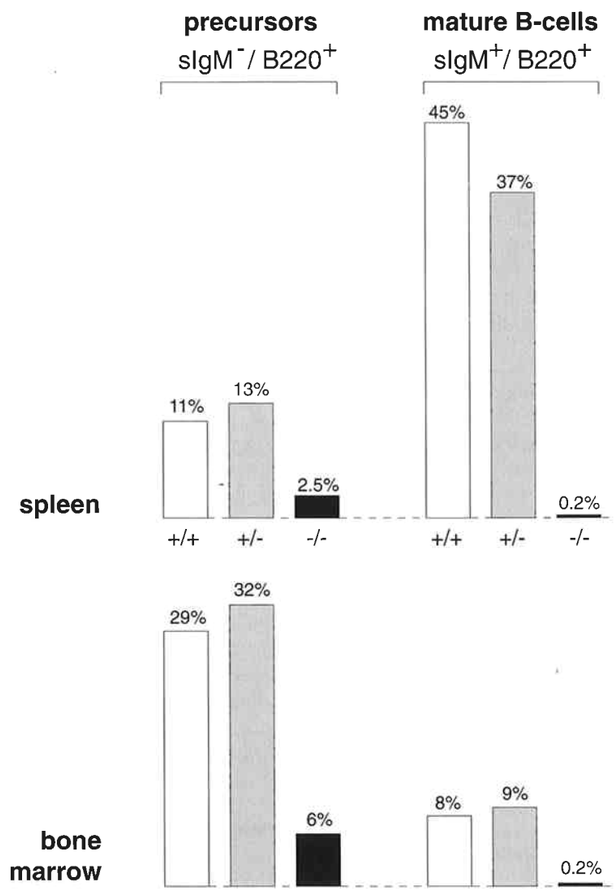

Fig. 4: Early block of B cell differentiation in mice lacking Pax-5.
The relative proportion of $sIgM^-$ and $sIgM^+$ B lymphocytes in spleen and bone marrow of 2-week-old mice was determined by flow cytometric analysis with anti-IgM and anti-B220 antibodies. For details see Urbánek *et al.* (1994).

Deregulated expression of PAX-5 expression in medulloblastoma

Zbynek Kozmik in collaboration with Adriano Aguzzi (University of Zürich)

Medulloblastoma is the most common pediatric brain tumor which typically arises in the cerebellum of children and young adults. This tumor is thought to originate from undifferentiated precursor cells present in the external granular layer of the embryonic cerebellum which normally migrate inwards during the first two years of life to give rise to the differentiated neurons of the internal granular layer. To date little is known about the molecular mechanisms underlying the pathogenesis of medulloblastoma. The identification of regulatory genes which are expressed in this pediatric brain tumor may therefore provide some insight into the molecular aspects of tumor formation. Members of the *PAX* gene family are potential candidate genes for two reasons. First, these transcription factors are known to be involved in patterning of particular brain structures (see above). Second, *PAX* genes have been implicated as oncogenes by in vitro transformation studies (Maulbecker and Gruss, 1993) and by the involvement of *PAX-3* and *PAX-7* in the genesis of alveolar rhabdomyosarcoma, a myogenic tumor of childhood (Davis *et al.*, 1994).

For these reasons we have analyzed a large collection of medulloblastomas for expression of the developmental control genes of the *PAX* and *EN* gene families by RNase protection and in situ hybridization (Kozmik *et al.*, 1995). Of all nine *PAX* genes investigat-

ed, only *PAX-5* and *PAX-6* were consistently expressed in most medulloblastomas (70% and 78% of all cases), as were the homeobox-containing genes *EN-1* (57%) and *EN-2* (78%) (see **Tab. 1**). The *EN-1*, *EN-2* and *PAX-6* genes were also expressed in normal cerebellar tissue, and their expression in medulloblastoma is consistent with the hypothesis that this tumor originates in the external granular layer of the developing cerebellum. *PAX-5* transcripts were, however, not detected in the cerebellum, indicating that this gene is deregulated in medulloblastoma. In the desmoplastic variant of medulloblastoma, *PAX-5* expression was restricted to the reticulin-producing, proliferating tumor areas containing undifferentiated cells, while *PAX-5* was not expressed in the reticulin-free islands consisting of differentiated cells. These data indicate that deregulated expression of *PAX-5* correlates positively with cell proliferation and inversely with neuronal differentiation in desmoplastic medulloblastoma. This observation may reflect a mere epiphenomenon. Alternatively, *PAX-5* may be causally involved in the establishment and/or maintenance of the neoplastic phenotype. We are currently testing this latter hypothesis by targeting expression of the *PAX-5* gene to the appropriate cerebellar compartment in transgenic mice.

gene	expression in medulloblastoma	expression in cerebellum
PAX-1	8%	-
PAX-2	0%	+
PAX-3	30%	++
PAX-4	0%	-
PAX-5	70%	-
PAX-6	78%	+++
PAX-7	0%	-
PAX-8	0%	-
PAX-9	0%	-
EN-1	57%	++
EN-2	78%	+++

Tab. 1: Expression of *PAX* and *EN* genes in medulloblastoma.
The frequency with which individual genes were expressed in a large collection of medulloblastoma is indicated together with the expression level in the normal cerebellum. For details see Kozmik *et al.* (1995).

2) Regulation of gene expression by c-Fos/AP-1

Introduction

Eukaryotic cells communicate via extracellular signals that interact with specific cell surface receptors and thus initiate a cascade of biochemical events leading to selective regulation of gene expression. The transcription factor AP-1 is thought to play a central role in reprogramming of gene expression in response to signal transduction. AP-1 is a complex mixture of polypeptides consisting of three Jun proteins (c-Jun, JunB and JunD) and four Fos proteins (c-Fos, FosB, Fra-1 and Fra-2). Jun proteins are able to form homo- and heterodimers, while Fos proteins are only capable of forming heterodimeric complexes with Jun proteins. Both Jun and Fos contribute to the transactivation function of the AP-1 complex, which stimulates transcription by binding to AP-1 recognition sequences (TGA G/C TCA) in enhancer and promoter regions (reviewed by Angel and Karin, 1991).

AP-1 has been implicated in diverse cellular processes including cell proliferation, differentiation and neuronal function. The role of the different members of the AP-1 genes in these processes have recently been addressed by gene inactivation experiments (see report by Erwin Wagner). Some insight into the molecular mechanisms of AP-1 function has also been obtained by the identification of genes that are controlled by this transcription factor. To date, >20 different AP-1 target genes have been characterized by defining functional AP-1 elements in their control regions using in vitro mutagenesis and transient transfection experiments (reviewed by Busslinger and Bergers, 1994). As an alternative approach to the identification of AP-1 target genes we have developed estrogen-dependent transcriptional and posttranslational Fos induction systems which allow selective activation of AP-1 by providing the limiting Fos component to cycling cells (Braselmann *et al.*, 1993; Superti-Furga *et al.*, 1991).

These induction systems were successfully applied to the identification and characterization of several genes which are up-regulated by AP-1 activity in Rat-1A fibroblasts and pheochromocytoma PC12 cells (Busslinger and Bergers, 1994). One of these genes is *fra-1*, a member of the AP-1 gene family itself. In the last year we have completed the identification and characterization of regulatory sequences in the *fra-1* gene which mediate stimulation by Fos/AP-1

Intragenic sequences in the 5' region of the fra-1 gene mediate transcriptional stimulation by Fos/AP-1

Paula Graninger and Gabriele Bergers

Constitutive expression of c-Fos or other members of the AP-1 family (FosB, Fra-1 and c-Jun) leads to up-regulation of the immediate-early gene *fra-1* in rat fibroblasts. Using the posttranslational FosER induction system (Superti-Furga *et al.*, 1991), we have demonstrated that stimulation of *fra-1* expression by Fos/AP-1 is a general phenomenon, as it is also observed in PC12 and mammary epithelial cell lines. Induction of *fra-1* transcription is rapid in response to FosER activation, and direct regulation was demonstrated by analyzing chimaeric *fra-1/β-globin* genes in FosER-expressing fibroblasts (**Fig. 5**). While 5' flanking sequences up to -3700 were unable to confer regulation by AP-1, we found that transcriptional stimulation was mediated by *fra-1* sequences in exon 1 and intron 1. These sequences contain three functional AP-1 binding sites which appear to mediate up-regulation of the *fra-1* gene by AP-1 activity (**Fig. 5**). Transactivation studies indicated that Fra-1 differs from c-Fos in its transcriptional properties. Fusion proteins consisting of full-length Fra-1 linked to the DNA-binding domain of Gal4 were unable to transactivate a Gal4-responsive promoter in contrast to the respective c-Fos fusion proteins. This finding suggests that Fra-1 may limit the activity of other Fos proteins at later times during serum stimulation by sequestering Jun partner proteins into less active AP-1 complexes. Fra-1 also possesses oncogenic potential despite the lack of a transactivation domain, as constitutive expression of Fra-1 conferred anchorage-independent growth to Rat-1A fibroblasts and resulted in tumor formation in nude mice. Hence, the *fra-1* gene is not only induced by c-Fos activity, but its protein may also contribute to cellular transformation elicited by deregulated c-Fos expression. All these data identify *fra-1* as a unique member of the *fos* gene family which is under positive feedback control by AP-1 activity (Bergers *et al.*, 1994).

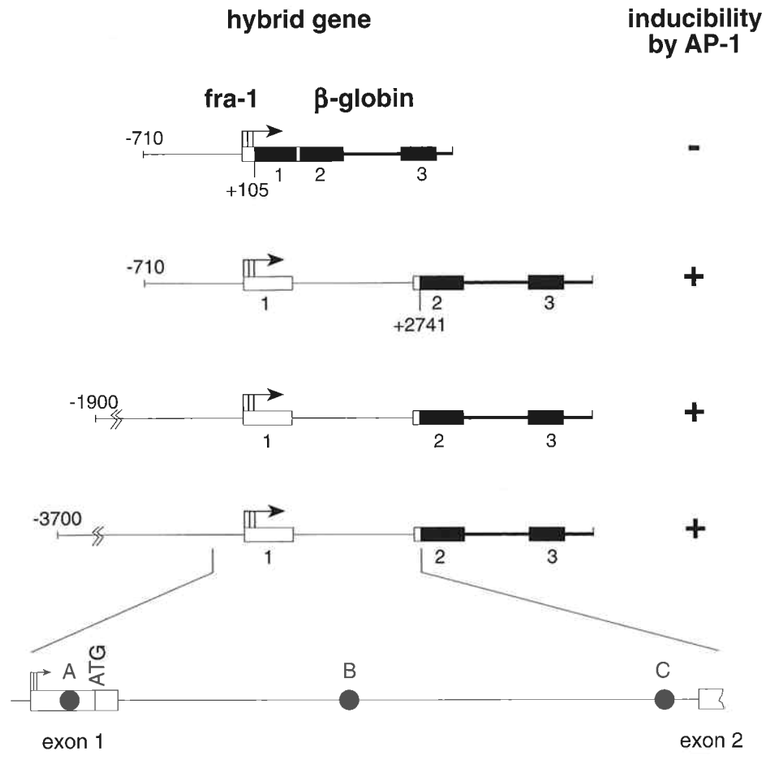

Fig. 5: Sequences in the 5' region of the *fra-1* gene mediate induction by Fos/AP-1.
Transcriptional stimulation of the indicated chimaeric genes by AP-1 was analyzed in FosER-expressing rat fibroblasts. The positions and sequences of the three AP-1 sites in exon 1 and intron 1 of the *fra-1* gene are shown below. For details see Bergers *et al.* (1995).

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The Yeast Cell Cycle

Group leader	Kim NASMYTH
Postdoc	Christian KOCH
Postdoc	Etienne SCHWOB
Postdoc	Manfred NEUBERG (until July 94)
Postdoc	Celia DOWZER (until Sept. 94)
Postdoc	Thomas BÖHM
Postdoc	Stefan IRNIGER
Postdoc	Simonetta PIATTI
Postdoc	Christine MICHAELIS (since May 94)
Postdoc	Wolfgang ZACHARIAE
Postdoc	Nicoletta BOBOLA (since Oct. 94)
Postdoc (ext.)	Ralf Jansen (since Nov. 94)
PhD Student	Fatima CVRCKOVÁ
PhD Student	Robert SIEGMUND
PhD Student	Christian DAHMANN
PhD Student	Dunja KNAPP
PhD Student	Isabel GONZALEZ (since Oct. 94)
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 kinases 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 triggers the initiation of DNA replication, whereas activation by G2-specific cyclins in G2 causes entry into mitosis. This year, we have made important progress 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, how cells prevent re-replication during G2, and how proteolysis of mitotic cyclins is regulated.

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 daughter cell. Only mother cells express the *HO* endonuclease gene and thereby switch their mating type. We have recently identified a new protein kinase essential for the generation of daughter cells and six new genes needed for the mother cell specific transcription of *HO*.

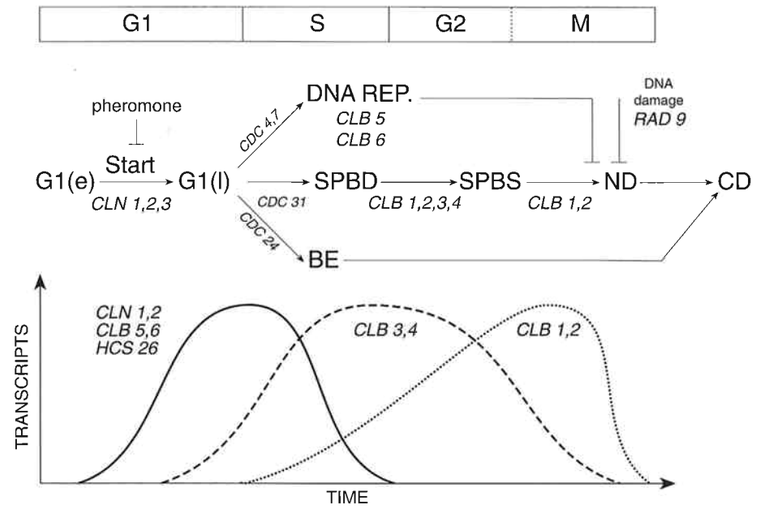

Fig. 1: The abundance of cyclin transcripts and their functions during the cell cycle of *S. cerevisiae*. In almost all cases, CDC28 kinase activity associated with cyclin proteins fluctuates in a manner similar to their transcripts. SPBD, spindle pole body duplication; SPBS, spindle pole body separation (i.e. the formation of bipolar spindles).

Starting the cell cycle

The role of gene activation in late G1

L. Dirick, R. Siegmund, M. Neuberg, and C. Koch

When yeast cells reach a critical size in late G1 (**Fig. 2**) they simultaneously start budding, initiate DNA synthesis, duplicate spindle pole bodies (**Fig. 3**) and activate a transcription program that leads to the expression of late G1-specific RNAs including those for the G1 cyclins Cln1 and Cln2 and for many DNA synthesis genes (**Fig. 4**). These different events, which have collectively been called "START" require an active Cdc28 kinase to be associated with at least one of the three G1 cyclins Cln1, Cln2 and Cln3.

The finding that transcription of *CLN1* and *CLN2* is dependent on an active Cdc28/Cln kinase led to the proposal of a positive feedback loop model, whereby Clns activate their own transcription through their involvement in the Cdc28/Cln kinase. This model predicts that functional Cln1 and Cln2 proteins are required for proper *CLN1* and *CLN2* transcription. We have now directly tested this prediction and surprisingly, we find that Cln1 and Cln2 are not required at all for their own transcription, disproving the positive feedback loop model.

In contrast to Cln1 and Cln2, Cln3 whose protein levels do not greatly change during the cell cycle, is essential for the proper cell size-dependent activation of all late G1-specific transcription, including *CLN1* and *CLN2* RNAs. The three Clns therefore have specialized functions for the start of the cell cycle. Cln3 activates transcription of genes in late G1 (**Fig. 4**), whereas Clns 1 and 2 shut off proteolysis of mitotic cyclins, trigger the disappearance of a B-type cyclin inhibitor p40Sic1 and thereby promote S phase, and promote bud formation.

Expression of *CLN1*, *CLN2* and of DNA synthesis genes is dependent on two related heteromeric transcription factors, SBF and MBF. SBF is composed of Swi4 and Swi6 and binds to the promoters of *CLN1* and *CLN2*, while MBF, a complex of Mbp1 and Swi6 binds to promoters of DNA synthesis genes (**Fig. 4**). While activation of late G1-specific transcription is dependent on Cdc28/Cln kinases, repression of SBF-dependent transcription during G2 requires a functional Cdc28/Clb kinase. We have shown that Clb2 kinase binds directly to a defined region of Swi4, called the ankyrin repeats. Interestingly, the ankyrin repeats have also been found as a motif in CDK inhibitors from *D. melanogaster* and humans.

Finally, we find that overexpression of Swi4 in strains lacking Mbp1 causes cells to divide at very small cell sizes, even in the absence of *CLN3* function. This is consistent with the notion that *CLN3*'s only function in starting the cell cycle is to activate transcription regulated by SBF and MBF.

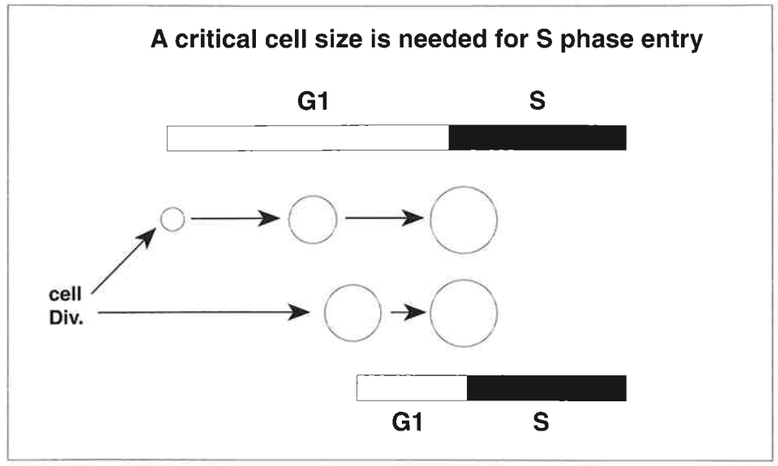

Fig. 2: Small daughter cells must spend longer than larger mother cells growing large enough to initiate DNA replication.

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 (**Fig. 5**). *CDC34* encodes an ubiquitin conjugating enzyme and *CDC4* encodes a protein that contains multiple copies of a motif found in β-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

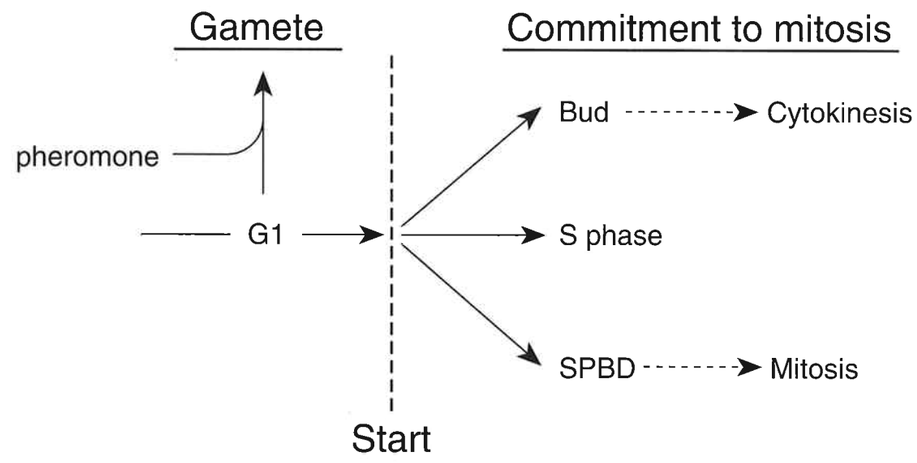

Fig. 3: When cells of the budding yeast *Saccharomyces cerevisiae* reach the critical cell size, they not only enter S phase but also initiate cytokinesis by forming buds, initiate the assembly of the mitotic apparatus by duplicating spindle pole bodies, and acquire resistance to pheromones that arrest progression through late G1. This point in the cell cycle is known as Start.

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* (see **Fig. 1**). 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 now show 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. We show that 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 in mutants that lack p40. Thus, activation of Clb/Cdc28 kinases through the proteolysis of p40^{SIC1} inhibitor may be an important step for the initiation of DNA replication in yeast (**Fig. 6**). *SIC1* mRNAs accumulate to high levels as cells exit from M phase due to transcriptional activation by Swi5.

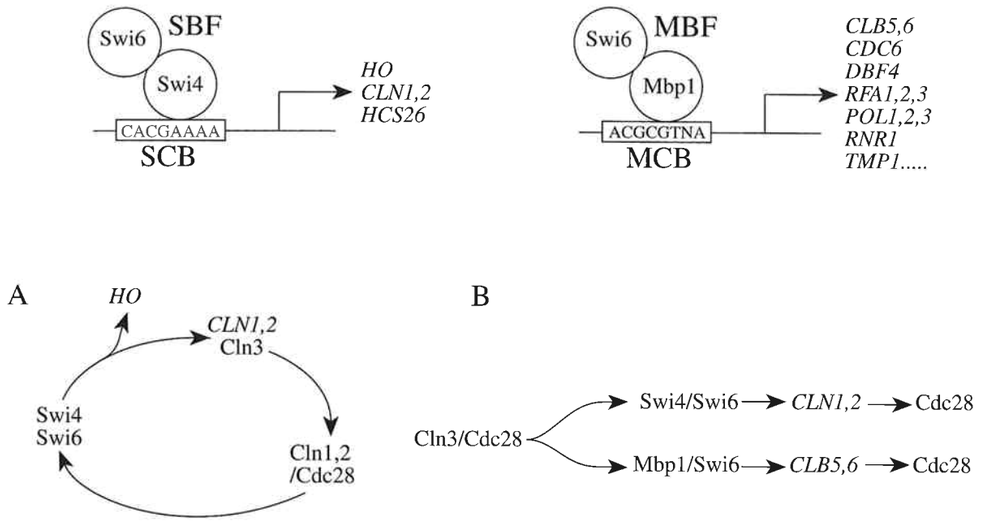

Fig. 4: The SBF and MBF transcription factors are responsible for late G1-specific expression of a number of genes involved in Start and DNA replication. SBF, SCB-Binding Factor; SCB, Swi4,6 cell Cycle Box; HO, mating type-switching endonuclease. MBF, MCB-Binding Factor; MCB, MluI Cycle Box; Mbp1, MluI-Binding Protein; RFA1,2,3 encode the three subunits of replication factor A (RPA); POL1,2,3, DNA polymerases; RNR1, ribonucleotide reductase; TMP1, thymidylate synthase.
Models for late G1 transcriptional activation. (A) Positive feedback loop; (B) linear model. The positive feedback hypothesis was invalidated by showing that Cln1,2 are not needed for their own expression; Cln3 is sufficient for full induction of CLN1,2 and CLB5,6 when cells reach the critical size for Start.

Ordering S-phase and mitosis in yeast

Christian Dahmann

Cells maintain a constant ploidy during proliferation because the duplication of chromosomes during S-phase is followed by their equational division and segregation at mitosis. S-phase and M-phase are triggered by S-phase promoting factor (SPF) and M-phase promoting factor (MPF), respectively. The mechanisms which ensure the strict alternation of S-phase and mitosis are still poorly understood. To address this question, we designed a genetic screen to identify mutants which would replicate their DNA twice per cell cycle.

A yeast cell lacking the B-type cyclins *CLB1-4* (*clb-ts* at restrictive temperature) is devoid of MPF and arrests with replicated DNA before mitosis. We have identified mutants (*sag*) which allow the *clb-ts* strain to undergo a second round of DNA replication in the absence of mitosis. These mutants furthermore form a second long bud which is indicative of a loss of Clb5,6 activities. Clb5,6 are two other B-type cyclins which are required for the timely occurrence of S-phase, i.e. they have SPF activity. Curiously, overexpression of *CLB5* suppresses the re-replication in *sag clb-ts* mutants. Based on this and other observations we propose a model in which SPF has two functions. One is to trigger S-phase and the second is to prevent re-initiation of DNA replication in a given cell cycle. Experiments are in progress to test this idea.

The mutants fall into four complementation groups *SAG1-4* (Start AGain). The corresponding wild type genes of *SAG1* and *SAG3* have been cloned. *SAG1* is a newly identified gene. Its gene product is highly homologous to two other yeast proteins: Nca3, which is involved in expression of a mitochondrial gene and Uth1, whose function is unknown. *SAG3* is identical to *SEC72*. Sec72 forms a complex with Sec63 and Sec71 and is required for protein translocation through the ER membrane. Sec63 has also been implicated in nuclear import, implicating that also Sec72 might have a role in nuclear import. It is currently not clear, how the *sag3/sec72 clb-ts* mutant gives rise to the re-replication phenotype.

In the future we would like to unravel the mechanisms by which SPF can act both as a trigger of S-phase and an inhibitor of re-replication.

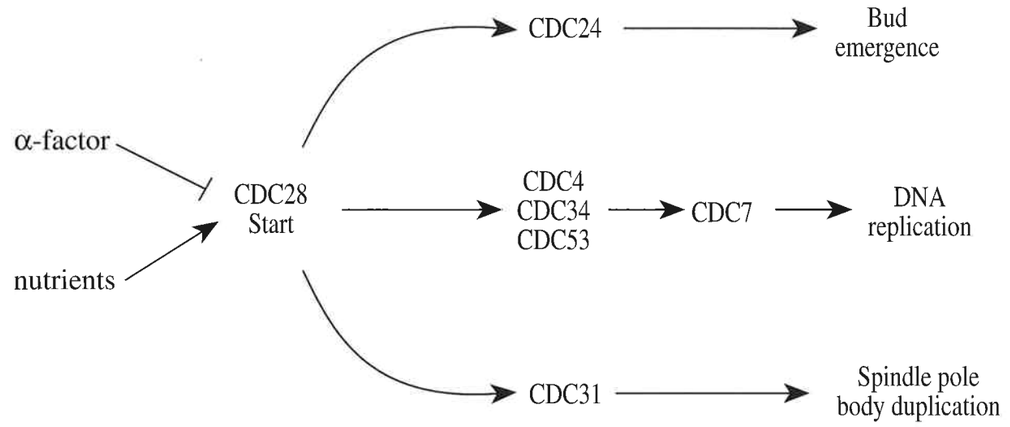

Fig. 5: The step controlled by Cdc28 is needed for all aspects of cell division, and is the focus of control by nutrients and pheromones. It was the earliest function in the sequence of dependent events, and therefore called the "Start" of the cell cycle. CDC4 along with CDC34 and CDC53 (not shown) are needed for S phase but neither for budding nor for spindle pole body duplication.

Cdc6 is an unstable protein whose *de novo* synthesis in G1 is important for the onset of S phase and for preventing a "reductional" anaphase in the budding yeast *Saccharomyces cerevisiae*

Simonetta Piatti

In most eukaryotic cells, DNA replication is confined to a narrow interval called S phase, it must be completed before cells can undergo nuclear division, and it cannot be repeated until cells have completed mitosis. Cell fusion experiments, performed over twenty years ago, suggested that a cell's ability to enter S phase is determined by the state of both nucleus and cytoplasm. Fusion of G1 or G2 cells with S phase cells induced chromosome duplication in G1 but not in G2 nuclei. This led to the proposal that S phase might be triggered by the accumulation in late G1 of an S phase promoting factor (SPF) that can induce a G1 but not a G2 nucleus to initiate chromosome duplication. Identifying SPFs and their nuclear targets has ever since been an important goal in cell cycle research.

Recent work suggests that cyclin dependent kinases (CDKs) play an important part in triggering S phase; that is, they correspond to SPFs. There is a hierarchy of S phase promoting factors in budding yeast. At its head resides Cln3, which activates synthesis of Clns1/2 and Clbs5/6. Next come Clns1 and 2, which trigger inactivation of P40Sic1 and thereby activate Clbs 5 and 6, which, it is supposed, phosphorylate key proteins at replication origins.

The targets of S phase promoting Clb/Cdc28 kinases are not known. Components of ORC (Origin Recognition Complex), a multiprotein complex that binds to origins of replication (ARSs) are possible candidates. Though ORC binds to origins throughout the cell cycle, in vivo footprinting suggests that ARS chromatin switches between a pre-replicative state characterisitic of G1 cells and a post-replicative state found during G2 and M phases. The pre-replicative state can be established at the end of mitosis or in late G1, and possibly at any time between exit from mitosis and the onset of S phase. DNA replication might be triggered by phosphorylation of proteins found only at origins in a pre-replicative state, which would explain why G2 nuclei do not respond to SPFs. In addition to ORC, other possible components of pre-replicative origins are proteins like Mcm5/Cdc46, Mcm2, Mcm3 and Cdc6, which are also required for S phase. The Cdc46/Mcm proteins form a family of related proteins, of which Cdc46p and Mcm3p accumulate in the cytoplasm during G2 and M phase and do not enter the nucleus till the end of mitosis; that is, when origins adopt a pre-replicative state.

Translation of G1 cyclins during G1 could account for the old observation that *de novo* protein synthesis is needed for S phase. It is less clear whether replication proteins that might be targets for Clb5/6 Cdc28 kinases must also be synthesized shortly before S phase. Many if not most of the DNA replication fork enzymes whose transcripts are regulated by MBF are stable proteins which are present in large excess and do not need to be synthesized *de novo* each cell cycle. Despite their movement in and out of the nucleus, Cdc46/Mcm proteins also do not fluctuate much in abundance during the cell cycle.

A good candidate for an unstable replication

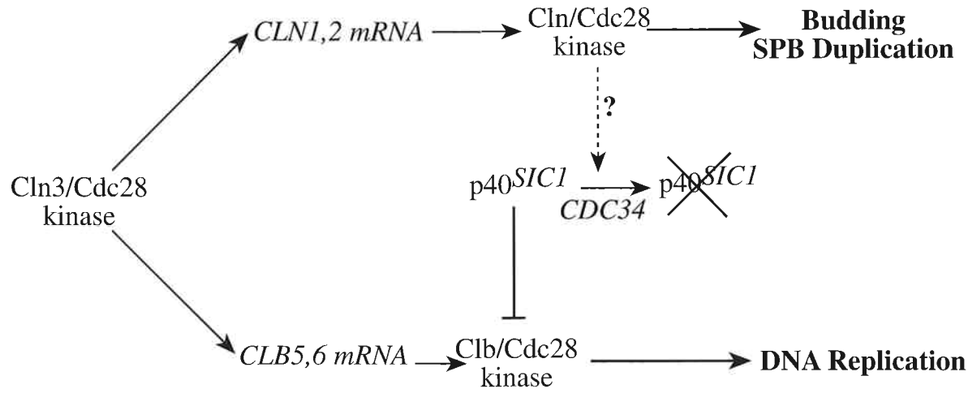

Fig. 6: Degradation of Sic1 is needed for S phase entry. DNA replication depends on the Clb5,6/Cdc28 kinase but is controlled by the Cln/Cdc28 kinase. Transcription of CLB5,6 requires Cln3 whereas Sic1 proteolysis, which is needed for activation of the Clb kinases, might require the Cln1,2/Cdc28 kinase.

protein whose synthesis in G1 may be needed for S phase is that encoded by *CDC6*. *cdc6* mutations affect the rate of DNA synthesis and have been reported to cause haploid cells to arrest with a 1N DNA content. Furthermore, the high rate of loss of a minichromosome in *cdc6-1* mutants can be suppressed by the addition of multiple ARS sequences, indicating that the Cdc6 protein acts at origins rather than at replication forks. The findings that *CDC6* mRNAs are cell cycle regulated and that overexpression from the *GAL* promoter but not from high copy plasmids causes cells to arrest in G2 led Bueno and Russell to suggest that Cdc6 might be an unstable protein. The fission yeast *Schizosaccharomyces pombe cdc18* gene encodes a protein that is 28% identical to Cdc6p and is also needed for DNA replication. Its transcription is activated transiently in late G1 by DSC1, the fission yeast homologue of MBF.

Most defects in DNA replication, whether caused by mutations or inhibitors, prevent a cell's entry into metaphase and/or anaphase. It is currently thought that while replication is incomplete, surveillance mechanisms emit signals that block the activity of M phase promoting Cdks. A number of budding yeast genes like *RAD9*, *RAD17*, *MEC1* and *MEC2* are necessary for this control, but their function and downstream targets are unknown. Another unanswered question is how cells sense whether replication is incomplete. *S. pombe* mutants with defects in *cdc18*, *cut5*, and *cdt1* enter M phase without undergoing S phase, which suggests that cells cannot recognize certain replication defects. It is possible that these mutants fail totally to assemble the structures that are recognized as replication intermediates.

We have addressed the timing and functions of Cdc6 synthesis. We show that Cdc6 is an unstable protein that is synthesized at the end of mitosis due to transcriptional activation by Swi5/Ace2 transcription factors. However, when a long G1 period intervenes before the onset of S phase, as in daughter cells, Cdc6 is re-synthesized in late G1 due mainly to transcriptional activation by MBF or SBF. We addressed the importance of these two modes of synthesis by expressing *CDC6* from the *MET3* promoter, which can be repressed by the addition of methionine. Daughter cells that had been allowed to synthesize Cdc6 during their exit from mitosis were able to enter S phase upon activation of Clb/Cdc28 kinases at the critical size but they were very slow to complete it, suggesting that the late G1 burst of synthesis performs an important function. Cells in which Cdc6 was synthesized neither as they exited from mitosis nor at any time during the subsequent G1 period failed to initiate DNA replication but nevertheless underwent nuclear division and thereby gave birth to mother and daughter cells with fractional DNA contents. Thus, budding yeast cells cannot recognize the replication defect due to an extreme Cdc6 deficiency, as is the case for Cdc18 in *S. pombe*. More remarkable still, we were able to demonstrate that haploid Cdc6-deficient cells undergo a "reductional" anaphase in which entire chromsomes are segregated either to one or the other spindle pole, an observation that sheds important light on the mechanism of mitosis, at least in yeast.

Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins at the beginning of the next cell cycle

Angelika Amon, Stefan Irniger

If we are to understand how the order of cell cycle events is determined, we need to understand the mechanisms that determine the periodicities of Cdc28 kinases associated with different cyclins. Cdc28 kinase activity associated with Cln1 and Cln2 is largely determined by control of *CLN1* and *CLN2* transcription. These genes are activated in late G1 by the SBF transcription factor, which is composed of the Swi4 and Swi6 proteins. Activation depends on Cdc28 and Cln3 function. Repression of *CLN1* and *CLN2* transcription as cells enter G2 depends on *CLB1-4* and might involve the physical association of SBF with Clb proteins. This transcriptional regulation ensures that Cdc28 kinase activity associated with the Cln1 and Cln2 proteins rises in late G1 and declines as cells enter G2.

Transcriptional controls could also have a role in determining the abundance of Cdc28 kinase activity associated with the B-type cyclins Clb1 and Clb2. Transcripts from *CLB1* and *CLB2* are absent in G1, rise as cells enter G2, peak just prior to anaphase, and drop as cells exit from mitosis. This pattern is similar to that of Cdc28 kinase activity associated with their gene products.

Post-translational as well as transcriptional controls have an important role in regulating Clb cyclin activity. B-type cyclins were originally discovered as proteins whose abundance oscillates during cleavage divisions of sea urchin embryos. In these cells, B-type cyclins are synthesized at a constant rate but destroyed suddenly during each mitosis. The proteolysis of B-type cyclins at mitosis is dependent upon a nine amino acid N-terminal sequence called the "cyclin destruction box", which is conserved in Clb1 and Clb2. It is therefore thought that the activation of Clb proteolysis at anaphase causes the destruction of Clb kinase activity and thereby, at least in yeast, the inactivation of *CLB* gene transcription. This cell cycle-dependent Clb proteolysis was thought to be confined to a narrow window during mitosis.

We have now shown that proteolysis mediated by the cyclin B destruction box is not in fact confined to mitotic cells but continues through the subsequent G1 period, until the activation of Cln cyclins. This finding could explain how the order of Cln and Clb cyclin oscillations is controlled during the yeast cell cycle (**Fig. 7**). Clb cyclins cannot accumulate before Cln cyclins, because the latter are required to inactivate proteolysis of the former. Cln cyclins do not possess cyclin destruction boxes and are therefore not subject to the same form of cell cycle-regulated proteolysis and as a consequence they can accumulate (at least transiently) upon the production of their mRNAs in late G1.

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

Stefan Irniger, 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 re-assemble (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

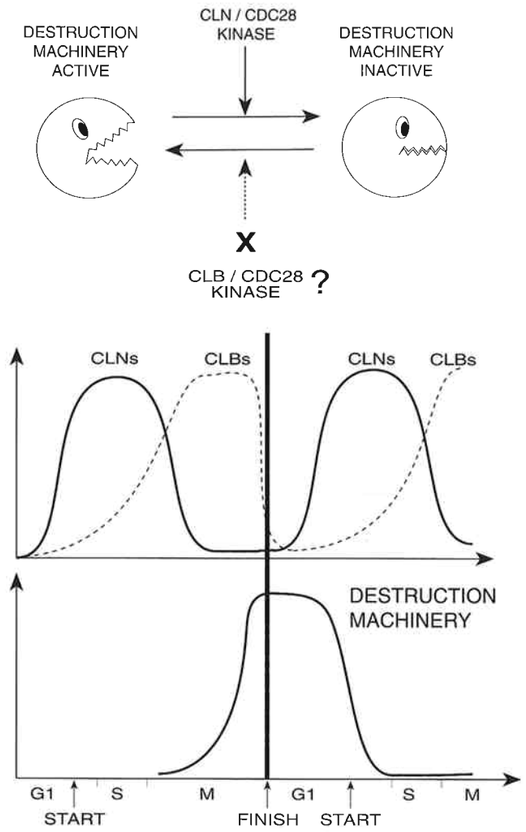

Fig. 7: Shutting off CLB2 proteolysis initiated during mitosis depends on activation of CLNs in the subsequent G1 period. This helps to determine the correct order of G1 and G2 cyclin oscillations.

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 re-activation 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. We have utilized this property to isolate mutants defective in Clb2 proteolysis. We identified 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 Cdc16p and Cdc23p may orchestrate the proteolysis during anaphase not only of mitotic cyclins but also of proteins whose degradation is needed for sister chromatid separation. We showed 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.

Cell morphogenesis and asymmetric gene expression

Ste20-like protein kinases are required for localized cell growth and cytokinesis in budding yeast

Fatima Cvrcková

Mechanisms of cytokinesis are extremely variable amongst eukaryotes. One variant of this theme is cell division by budding, typical for yeasts. The cleavage furrow in animal cells or the preprophase band (a cytoskeletal structure determining the future cell division plane) in plant cells form only after most of the growth required to double the mass has occurred, while in budding the formation of the "cleavage furrow" (bud neck) precedes the bulk of cell growth. Unlike cleavage or fission, budding is dependent on the ability to direct growth exclusively to one side of the division plane - to the bud. The inherent asymmetry of this process perhaps brings an evolutional advantage by facilitating the formation of variable progeny. In any case, it has proven quite successful in evolution, as documented by the diversity of yeasts and yeast-like fungi. Despite differences in timing and morphology, molecular mechanisms underlying cytokinesis seem to be similar for all forms of cell division: several proteins required for budding and cytokinesis in the yeast *Saccharomyces cerevisiae* have homologues in higher eukaryotes. Studies in yeast may be as useful for understanding cytokinesis and its regulation as they have been for elucidating the mechanisms of cell cycle control by cyclin-dependent protein kinases or signal transduction.

Like other cell cycle processes, the timing of cytokinesis must be carefully regulated. In yeast, the first step towards cytokinesis is bud emergence. Yeast cells bud in late G1, concurrently with the onset of DNA replication and with the duplication of the spindle pole body. The simultaneous onset of these three processes is often referred to as "start". The three aspects of "start" occur as a consequence of the activation of a cyclin-dependent protein kinase, Cdc28, by at least one of the G1 cyclins Cln1, Cln2 and Cln3. Under normal conditions, Cln3 induces the accumulation of Cln1 and Cln2 which then trigger budding and DNA replication.

Bud emergence results from several distinct events. The cell must choose a site on its surface (the bud site) and direct growth towards this site, ensuring at the same time that no other part of the cell exterior grows.

The first event necessary for budding - bud site establishment - requires the products of multiple genes including *CDC42*, which codes for a Rho-like GTPase that is localized at the bud site, *CDC24*, coding for a GDP/GTP exchange factor and *CDC43*, encoding a farnesylation enzyme required to attach Cdc42 to membranes. A group of non-essential genes (*BUD1* to *BUD5*) is responsible for non-random localization of the bud site with respect to the previous bud. One of them, *BUD2*, is essential for budding in cells lacking Cln1 and Cln2, suggesting that bud emergence is controlled by Cln1 and Cln2.

A second prerequisite for budding is a change in cell growth mode that occurs at "start". Pre-start cells grow in a non-polar manner, increasing their size but maintaining their shape. After "start", growth becomes limited to a part of the cell (the nascent bud). This polarization of growth is accompanied by translocation of cortical actin to the growing area, triggered by Cdc28 and Cln1 or Cln2. Overexpression of Cln1 or Cln2 causes "hyperpolarized" growth, suggesting that Cln1 and Cln2 have a role in directing growth towards the bud site (or towards bud tip in budded cells). Later in the cell cycle, Cln cyclins are replaced by the mitotic B-cyclins. This is accompanied by the return to the non-polar growth mode that persists till next "start".

The third prerequisite of normal bud development is the establishment of a "border" between the bud and the mother cell and restriction of surface growth to the bud. Unlike the previous steps that are required not only for budding, but also for the morphological changes ("shmooing") in haploid cells differentiating into gametes, border formation is specific for budding. The border is marked by a ring of 10 nm filaments attached

to the cytoplasmic membrane. This ring forms around the bud site at the time of bud emergence and persists at the bud neck till cytokinesis. Mutants in *CDC3*, *10*, *11* and *12* fail to assemble the ring, produce malformed buds and cannot undergo cytokinesis. *CDC3*, *10*, *11* and *12* encode structural components of the ring, which are proteins closely related to each other, to products of several anonymous mammalian cDNAs and to the Drosophila *peanut* (Pnut) protein required for cytoplasmic division. Members of this protein family were collectively termed "septins".

Other proteins involved in budding are also evolutionarily conserved. Homologues of Cdc42 have been found in higher eukaryotes. Their biological function remains largely unknown; expression of mutationally activated Cdc42 in fibroblasts may cause a cytokinesis defect. Biochemical partners of Cdc42 in mammalian brain tissue were found. One of these partners, the Pak protein kinase, is related to a yeast protein - Ste20 - necessary for the response of haploid cells to sexual pheromones. Ste20 transduces the signal elicited by binding of pheromone to a membrane receptor towards a protein kinase cascade consisting of Ste11, Ste7 and Fus3/Kss1, one of several known yeast homologues of the mammalian MAPK system. Induction of the kinase cascade leads to transcriptional activation of pheromone-regulated genes and to cell cycle arrest in G1 as a prelude to conjugation. Loss of Ste20 or any other component of the pheromone response pathway does not affect the vegetative cell cycle in haploids. The interaction between the mammalian Ste20 and Cdc42 homologues was therefore surprising, since the known function of Ste20 does not indicate a role in budding or establishment of cell polarity. There is so far only one observation suggesting an involvement of Ste20 in morphogenesis. Ste20 and some other components of the pheromone response pathway are required for the formation of pseudomycelia in nitrogen-starved diploid cells. It is, however, unclear whether Ste20 functions in cell morphogenesis or in perceiving nutrient conditions during pseudomycelial differentiation.

How is budding linked to the rest of the cell cycle? We previously described the isolation of mutants defective in bud emergence only in the absence of Cln1 and Cln2 and proposed that Cln1 and Cln2 are involved in bud site establishment. The same genetic screen yielded mutants unable to undergo cytokinesis due to a defect in bud neck morphogenesis, suggesting that Cln1 and Cln2 have a role also in determining the border between the mother cell and its bud. One of the genes required for cytokinesis in *cln1 cln2* cells, *CLA10*, is allelic to *CDC12*, suggesting that septin ring assembly or function may be controlled by Cln1 and Cln2. Other mutants with a similar phenotype define a novel gene, *CLA4*, that codes for a putative protein kinase related to Ste20 and Pak. Similar to Pak, Cla4 can bind Cdc42. *CLA4* is a non-essential gene but Cla4 and Ste20 share an essential function required for the restriction of cell growth to the portion of cell surface enclosed by the septin ring. The phenotype of *cla4 ste20* double mutants identifies not only a role for Ste20-like protein kinases in morphogenesis but also a novel step in bud development.

Symmetric expression of the *HO* endonuclease in mother and daughter cells

Ralf Jansen

The result of a normal mitotic cell division are two cells of equal function and fate. During development, however, many divisions are asymmetric, giving rise to cells that undergo various processes of differentiation. The mechanisms that generate asymmetric cell fates are poorly understood.

One of the best characterized examples of asymmetric gene activation occurs during mating type switching in *S. cerevisiae*. In this budding yeast, switching takes place after a cell division in the larger mother cell but not in the smaller daughter cell. Switching is initiated by the enzymatic activity of the *HO* gene product, an endonuclease that introduces a double strand break at the mating type locus. *HO* is expressed only in mother cells and only at a certain window during the cell cycle, at START. Understanding the molecular basis for mother cell specific transcription of *HO* in yeast may provide clues as to how cell division gives rise to cells with different fates in other eukaryotes as well.

Till now, 10 genes (the so called *SWI* genes) have been identified that are necessary for *HO* expression. Only one of these, *SWI5*, has been shown to be necessary for the mother-specific expression of *HO*. However, *SWI5* alone is not sufficient for asymmetric *HO* activation. We have designed a new screen to identify novel genes involved in the control of mother-specific *HO* expression, which allowed us to exclude easily previously identified *SWI* genes.

So far, mutants in 6 new complementation groups/genes (called *SHE1*-6) have been identified that fail to express the *HO* gene. These mutations reduce mating type switching of mother cells to less than 5% (wild type: 60-70%). We could show that the genes affected in these mutants act on *HO* most via SWI5. The *SHE* genes are specific for *HO* since they do not affect the expression of at least one other Swi5-regulated gene, *EGT2*.

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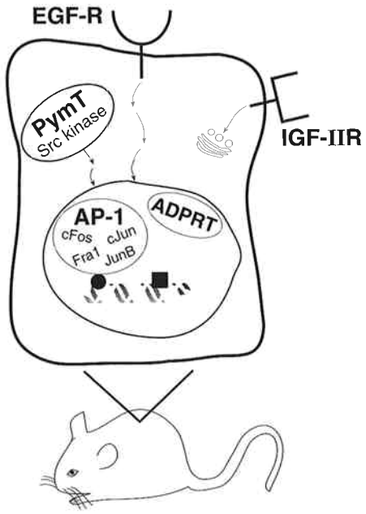
Gene Function in Mammalian Development and Oncogenesis

Senior scientist	Erwin F. WAGNER
Staff scientist	Zhao-Qi WANG
Postdoc	Agi GRIGORIADIS (until Nov. 94)
Postdoc	Maria SIBILIA
Postdoc	Kanaga SABAPATHY (since Aug. 94)
Visiting postdoc	Marina SCHORPP (since Aug. 94)
Technician	Ingrid FETKA
Technician	Laura STINGL
Technician	Uta MÖHLE-STEINLEIN (50%)
Technician	Jun LIANG (50%)
PhD student	Martin SCHREIBER
PhD student	Axel BEHRENS (since Nov. 94)
Diploma student	Anne-Karina PERL (until June 94)

Introduction



Transgenic mice
ES mice and Chimeras
Bone marrow reconstituted mice

Fig. 1: Overview of research projects

Growth control genes such as 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 (**Fig. 1**). We are interested in the function of such genes in normal mouse development, stem cell differentiation and tumor formation. Our approaches are aimed at eventually defining the function of specific gene products in the context of the whole organism. For these studies we are employing "gain-of-function" (overexpression) and "loss-of-function" (gene inactivation) experiments and are using as powerful tools: (i) Transgenic mice produced by micro-injection of DNA into fertilized eggs; (ii) genetically-altered mouse embryonic stem (ES) cells introduced into mouse embryos and (iii) retroviral gene transfer into hematopoietic cells.

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. AP-1 is composed of dimeric complexes formed between three Jun family members (c-Jun, JunB and JunD) and four Fos family members (c-Fos, FosB, Fra-1 and Fra-2) and is believed to play an important role in the regulation of cell proliferation, differentiation and gene expression. Our past efforts have focused on using gain-of-function and loss-of-function approaches for c-*fos* and c-*jun* (see below). We have broadened these studies to include other AP-1 family members, such as *fra*-1 and *jun*B and preliminary data from transgenic as well as from knockout experiments will be reported.

1. Analysis of c-Fos mutant mice

Agi Grigoriadis, Zhao-Qi Wang and Jun Liang

Defining the defects in Fos-less mice. Mice lacking the c-Fos proto-oncogene/transcription factor develop the bone remodeling disease osteopetrosis (Wang *et al.*, 1992). We investigated the cellular defect underlying this phenotype and demonstrated recently that c-Fos mutant mice are osteopetrotic because they lack bone-resorbing osteoclasts. Several lines of evidence, including rescue of the osteopetrosis by bone marrow cell transplantation demonstrated that the block in osteoclast progenitor cell differentiation is intrinsic to the osteoclast lineage rather than due to the stromal environment. In collaboration with Marco Cecchini and Herbert Fleisch (University of Berne) we showed that the lack of c-Fos also resulted in an increase in the number of tissue macrophages specifically in the bone marrow which are related to osteoclasts. These results suggest that Fos is an important regulator of both osteoclast and macrophage differentiation (**Fig. 2**) thereby identifying the cell types *in vivo* in which c-Fos function is essential.

To investigate whether the observed defects in osteoclast differentiation were due to the absence of c-Fos itself, we performed rescue experiments utilizing ectopic c-*fos* expression both *in vitro* and *in vivo*. Osteoclast differentiation and activity *in vitro* was assessed in co-cultures of spleen cells and osteoblastic cells following infection of spleen cells with a c-Fos retrovirus. Whereas spleen cells lacking c-Fos are unable to form osteoclasts when cultured with osteoblastic cells, infection of mutant spleen cells with a c-Fos virus relieved the block in osteoclast differentiation and restored functional osteoclastic resorption *in vitro*. In addition, ectopic expression of the *fos*-related gene, FosB, but not c-Jun, rescued in part osteoclast formation *in vitro*. A genetic rescue *in vivo* was performed by crossing c-Fos mutant mice with c-*fos* overexpressing transgenic mice. The expression of the c-*fos* transgene in mutant mice was able to rescue the osteopetrosis, but only after ~1 year. An increased bone marrow space was evident from 8.5 months of age in the mid-diaphyses of the long bones which became fully expanded by 13 months of age. Preliminary evidence also suggests that spleen cells from the rescued mice regained their ability to form osteoclasts *in vitro*. These results suggest that the observed defects in mutant mice are due to the lack of c-Fos and future experiments will aim to define the molecular mechanismof c-Fos in normal osteoclast and monocyte/macrophage differentiation.

AP-1 cooperativity in c-Fos-induced osteosarcoma formation. We have previously generated transgenic mice overexpressing the c-*fos* proto-oncogene and have demonstrated that ectopic c-*fos* expression leads to the development of bone tumors due to specific transformation of bone-forming osteoblastic cells (Grigoriadis *et al.*, 1993). In contrast, transgenic mice expressing c-*jun* from the same regulatory elements do not develop any pathology despite high expression in bone tissues. We investigated whether ectopic expression of both Fos and Jun would cooperate *in vivo* and possibly alter Fos-induced osteosarcoma formation. Double transgenic mice obtained by crossing c-*fos* and c-*jun* transgenic mice develop osteosarcomas at a higher frequency and at a faster rate than single Fos-transgenic mice, although there were no apparent differences in the time of onset of tumor formation. Histological analysis indicated that Fos-Jun tumors contained greater quantities of bone. Moreover, Northern blot analysis of isolated cell lines from Fos-Jun osteosarcomas indicated that while all osteoblastic genes were expressed, levels of the AP-1 responsive gene collagenase were apparently enhanced when compared to cell lines isolated from Fos-induced osteosarcomas. These studies indicate that ectopic expression of Jun can enhance Fos-induced oncogenesis *in vivo* and further highlight the importance of putative AP-1-responsive genes as regulators of osteosarcoma formation.

To define further the role of c-Fos in osteosarcoma formation, we also crossed c-*fos* transgenic mice with Fos mutant mice and generated transgenic mice lacking endogenous c-*fos* but carrying a c-*fos* transgene. Interestingly, there was a strong correlation between the frequency of osteosarcoma formation and the dosage of the endogenous c-*fos* gene. Osteosarcoma formation was reduced in c-*fos* +/- mice and almost absent in c-*fos* -/- mice carrying a c-*fos* transgene. These results suggest an important role for the

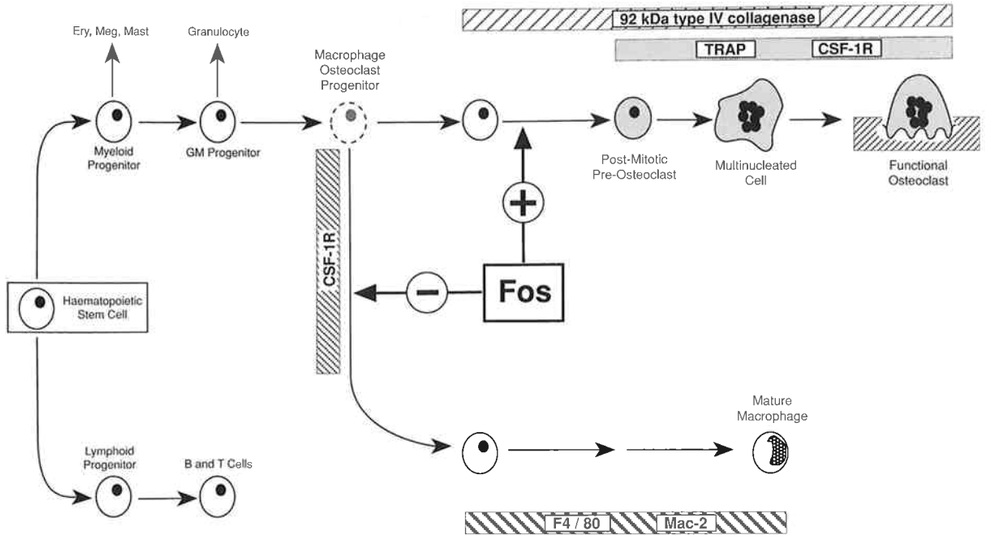


Fig.2: Model for c-Fos function in osteoclast-macrophage differentiation.

endogenous c-*fos* gene in initiating osteosarcoma formation in transgenic mice. We are currently investigating whether threshold levels of Fos protein or differences in protein modification between exogenous and endogenous Fos in osteoblastic cells are responsible for the observed effect.

2. The function of c-Jun in mouse development and hepatogenesis

Jun Liang and Ingrid Fetka in collaboration with Frank Hilberg (Bender & Co.), Karl Schellander (Vet. Univ., Vienna) and Kurt Zatloukal (Institute of Pathology, Graz)

Transgenic mice overexpressing c-*jun* do not develop any phenotype. In contrast, mice lacking c-Jun die at midgestation, implying an important function of c-Jun in normal mouse development (Hilberg *et al.* , 1993). Detailed analysis of adult chimaeras generated with c-*jun* -/- ES cells suggested that c-*jun* is an essential gene for hepatogenesis. To further investigate the role of c-Jun in liver development, expression analysis of several liver-specific genes was performed using RNA isolated from c-*jun*-/- embryos at different stages of gestation. Some liver-specific genes (e.g. HNF-1, -4) were found to be down regulated, although albumin and AFP were found to be expressed at similar levels as in heterozygous littermates, suggesting the presence of functional hepatocytes at mid-gestation. The capacity of c-Jun-/- cells to form hepatocytes was further investigated in several chimaeric mice generated with c-*jun*-/- ES cells. The distribution of differentiated mutant ES cells in hepatocytes during prenatal and postnatal development suggests that in the absence of c-Jun hepatocyte proliferation/differentiation is impaired at a critical stage after birth.

Through genetic complementation with c-*jun* transgenic mice (see Report 1993) we were able to rescue the embryonic lethality caused by the absence of c-Jun. A detailed analysis of the "rescued" c-Jun-/- mice expressing the H2-c-*jun* LTR transgene revealed a severe eye pathology and drastically reduced fertility. These data indicate that c-Jun has additional functions in other developmental processes, which are currently being analyzed.

3. Functional analysis of the murine *jun* B gene

Marina Schorpp and Zhao-Qi Wang in collaboration with Peter Angel (Genetic Institute, Karlsruhe)

JunB is an another important component of the AP-1 transcription factor complex and 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 employed once more gain-of-function and loss-of-function approaches by overexpressing the gene in transgenic mice and by inactivating the gene in ES cells and in mice.

Transgenic mice have been generated with the *jun*B gene expressed from the ubiquitin and H2 promoters. Northern blot analysis of embryonic and adult tissues revealed that the ubiquitin driven *jun*B transgene was expressed at high levels in all tissues as early as E10.5 days of gestation. Heterozygous transgenic mice overexpressing *jun*B do not exhibit any obvious phenotype and these mice are currently being used in genetic complementation experiments in an attempt to rescue the phenotype of c-*jun* and *jun*B knockout mice.

In the second approach the *jun*B gene was disrupted in ES cells and in mice. Heterozygous *jun*B mutant mice are phenotypically normal and fertile. However, no homozygous mutant newborns have been obtained from more than one hundred offspring genotyped from heterozygous intercrosses. Analysis of different stages of embryonic development showed that *jun*B-/- embryos die at ~E9.5. The phenotypic changes start to appear at ~E7.5 as most mutant embryos are growth retarded and seem to exhibit an altered forebrain region. Detailed histological analysis is being performed to examine which tissues are affected and *in situ* hybridization together with immunohistochemical studies using specific lineage markers will better define the function of *jun*B in mouse development.

4. Fra-1 is essential for normal mouse development

Martin Schreiber and Zhao-Qi Wang

Fra-1 is a member of the c-Fos gene family and is expressed at low levels in most organs in the adult mouse. To define the function of Fra-1 in mouse development, we have inactivated the *fra*-1 gene by homologous recombination in ES cells. For this purpose we have replaced the essential DNA binding and dimerization domains of *fra*-1 with an in-frame lacZ gene and a neomycin resistance gene. Fra-1+/- ES clones derived from several ES cell lines were used to generate chimaeric mice and also "ES mice", all of which transmitted the *fra*-1 targeted allele to their offspring.

Heterozygous *fra*-1+/- mice are phenotypically normal and fertile, whereas no *fra*-1-/- mice were obtained from more than 200 offspring analysed. Genotyping at various embryonic stages revealed that mutant embryos can first be identified as being phenotypically altered at ~E8.5. Thereafter, mutant embryos are severely growth retarded (**Fig. 3**). Embryos lacking Fra-1 die soon after E9.5 and preliminary analysis shows that these embryos seem to have phenotypic alterations in the developing hindbrain.

We have used the in-frame lacZ reporter gene integrated in the *fra*-1 locus to elucidate the temporal and spatial expression pattern of *fra*-1 during various stages of embryonic development. Whole mount histochemical analysis showed that expression of the *fra*-1 gene is widespread in the embryo and is apparently highest in the brain. Surprisingly, expression was detected at a significant level only in E10.5 and older embryos, but only very low, if any, expression was found at 8.5 and 9.5 days p.c., the stage when *fra*-1-/- embryos die. We are currently trying to identify the developmental lineage or process affected by the absence of *fra*-1, using histological analysis and whole mount *in situ* hybridization together with developmental marker genes. In addition, we are attempting to generate *fra*-1-/- ES cells and embryonic fibroblasts for further studies on the function of *fra*-1.

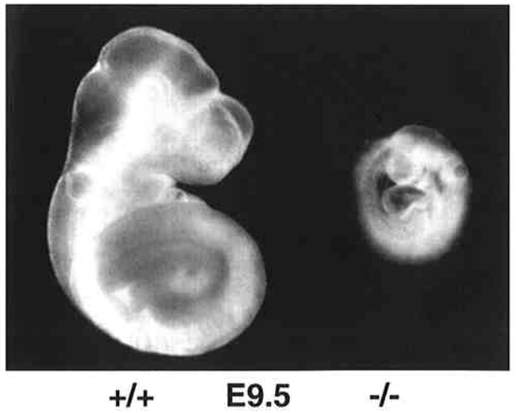


Fig.3: Mouse embryos lacking Fra-1 are severely growth retarded and die at day 9.5 of embryonic development.

5. The function of c-Jun and c-Fos in AP-1-dependent processes in fibroblasts

Martin Schreiber, Uta Möhle-Steinlein and Zhao-Qi Wang

To study the role of c-Fos and c-Jun in cell proliferation, transformation and gene expression we have used as a model system primary as well as 3T3-like fibroblasts from mouse embryos lacking either one or both of these proteins. In collaboration with Rolf Müller (IMT, Marburg) we have shown that proliferation of normally cycling cells and re-entry of quiescent cells into the cell cycle following serum stimulation are not c-Fos-dependent and occur with similar efficiency in c-*fos*-/- and control cells. We also demonstrated that there is no compensatory overexpression or activation of other known Fos or Jun family members. On the contrary, the c-*fos*-/- cells showed a reduced induction of *fra*-1 and collagenase I expression following serum or TPA stimulation, which is in agreement with the previous identification of these genes as c-Fos target genes. In Jun-less fibroblasts, the kinetics of induction of most AP-1 family member genes is delayed, and the peak induction level is often reduced indicating that c-Jun is an important regulator of AP-1 activity itself. Comparison of the AP-1 binding and transactivation activities by electrophoretic mobility antibody supershift and CAT assays suggests that c-Fos/c-Jun are not major components of AP-1 complexes in fibroblasts.

In contrast to Fos-less fibroblasts, both primary and 3T3-like fibroblasts lacking c-Jun show markedly reduced proliferation rates and saturation densities, which differs from the situation in ES cells lacking c-Jun. This proliferation defect cannot be rescued by coculture with wild-type fibroblasts. In spite of the dramatic proliferation defect in fibroblasts lacking c-Jun, the distribution of cycling cells in different phases of the cell cycle as well as the length of the G1- and S-phase of synchronized cells 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%. We are currently analyzing the molecular defects caused by the absence of c-Jun using several molecular markers as well as 2D gel analysis.

The rapid and pronounced activation of c-*jun* and c-*fos* transcription in UV-irradiated cells strongly suggests a role for these proteins in the mammalian UV-response. We have shown that cell survival after UV-irradiation is indeed significantly reduced in Jun-less and Fos-less cells. However, the ability to block cell cycle progression and to repair DNA damage upon UV-irradiation, both important protective mechanisms against the adverse effects of UV-light, are not affected in these cells, suggesting that a cellular target other than DNA is responsible for the UV-hypersensitivity of these cells. These cells as well as cells lacking other AP-1 family members should provide a powerful system to study where individual AP-1 proteins have unique biological functions and where they are largely redundant. Future experiments will attempt to determine the mechanism of UV-hypersensitivity of both Fos-less and Jun-less cells.

II. Studies on cells and mice lacking the NAD$^+$:ADP-ribosyl-transferase (ADPRT) gene

Zhao-Qi Wang and Laura Stingl in collaboration with Bernd Auer (University of Innsbruck) and Manfred Schweiger (University of Berlin)

ADPRT, the enzyme which catalyzes poly-ADP-ribosylation of nuclear proteins, is responsible for a major posttranslational modification process following DNA damage. Many studies have suggested a role for ADPRT in chromatin stability, DNA repair, cell proliferation, cell toxicity and also in human disease.

To elucidate the function of ADPRT *in vitro* and *in vivo*, the ADPRT gene was inactivated in ES cells and in mice. Mice lacking ADPRT are apparently healthy and fertile. Absence of ADPRT enzymatic activity in mutant tissues and lack of poly-ADP-ribose in ADPRT-/- embryonic fibroblasts suggest that ADPRT is dispensible during development and ADP-ribosylation is not an essential protein modification. DNA repair assays using mutant fibroblasts demonstrated that ADPRT is apparently not involved in DNA repair. However, the proliferation of mutant fibroblasts i*n vitro* is slower than that of wild-type fibroblasts. Cell cycle analysis was performed to further characterize the deficiency in fibroblast proliferation and preliminary results showed that there are no significant differences in cell cycle progression and re-entry into the cell cycle in the absence of ADPRT. Mutant thymocytes exhibit no differences in their apoptotic response to glucocorticoid hormones and gamma irradiation when compared to wild-type thymocytes, suggesting that ADPRT may not be involved in chromatin degeneration following induction of apoptosis. However, ADPRT-/- mice originating from a mixed genetic background (129/Sv x C57BL/6) are susceptible to skin disease, as older mice develop epidermal hyperplasia, spongiosis and parakeratosis (**Fig. 4**). In order to better understand the role of ADPRT in keratinocyte proliferation and differentiation, we exposed mutant skin to chemical carcinogens. Preliminary results show that mutant mice are less sensitive to TPA-induced papilloma

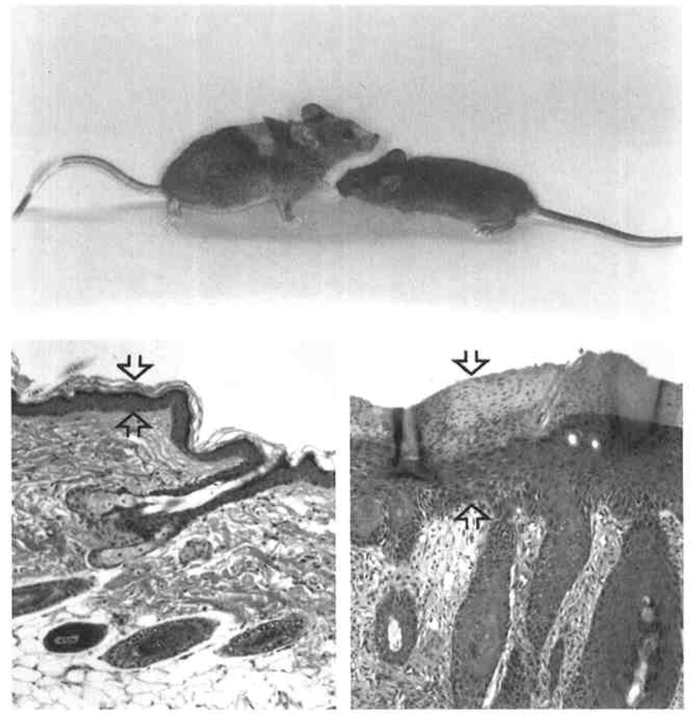


Fig. 4: ADPRT mutant mice exhibiting skin lesions.Top panel depicts an aged mutant ADPRT-/- mouse with the diseased skin (left) and a young healthy mutant mouse (right). Lower panels show a histological analysis of the skin lesions of an ADPRT-/- mouse with characteristic hyperplasia of the epidermis (arrows in right) compared to the normal architecture of the skin in a wild-type mouse (arrows in left).

formation. In addition, we are currently measuring the accumulation of chromosomal aberrations following exposure of cells to UV radiation and alkylating agents. The present analysis of ADPRT mutant mice suggests that ADPRT may function as a general protective mechanism for cellular responsiveness to environmental insults.

III. How does the PymT oncogene deregulate the proliferation of endothelial cells?

Uta Möhle-Steinlein, Ingrid Fetka and Kanaga Sabapathy

The middle T antigen of mouse 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. 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. End. cells form morphologically aberrant cyst-like structures in fibrin gels which, by addition of protease inhibitors, can be corrected into tubular structures like those formed by normal endothelial cells. To define the causal role of proteolysis in the formation of vascular lesions, mice lacking uPA, tPA and PAI-1 are being used. Preliminary data indicate that none of these components are absolutely required for deregulation of endothelial cell morphology (**Fig. 5**). In combination with inhibitor studies we are aiming to better define a causal role of proteolysis in PymT-induced vascular tumor development.

Since Src family tyrosine kinases are thought to be involved in mediating the oncogenic activity of PymT we investigated its ability to cause vascular lesions in c-*src*, c-*fyn*, and c-*yes* deficient mice. We demonstrated that the 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

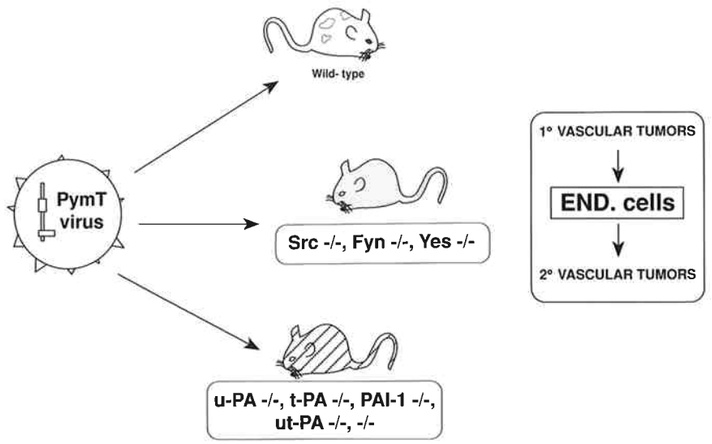


Fig.5: Molecular dissection of PymT action using knock out mice.

initiating action of PymT. We are presently analysing the potential of PymT to induce vascular tumors in double knock-out mice e.g. in *src-/-yes-/-* strains and are also testing the hypothesis that VEGF/Flk-1 may provide an essential component in the deregulation of endothelial cell growth. This work was performed in collaboration with Michael Pepper (Geneva) and Werner Risau (Bad Nauheim).

IV. Function of growth factor receptors

Growth factor receptors, in particular receptor tyrosine kinases, are known to play important roles in the regulation of cell proliferation and differentiation and their deregulated expression is often involved in the process of tumor formation. One of the best known members of the tyrosine kinase receptor family is the epidermal growth factor receptor (EGF-R), which is believed to have important functions in mouse development and cell differentiation. To gain better insights into the function of growth factor receptors, we have inactivated the EGF-R and, in collaboration with Denise Barlow, the insulin-like growth factor type 2 receptor *Igf2/MPR* in ES cells and mice.

1. Analysis of mice and cells lacking the EGF receptor

Maria Sibilia

The EGF-R is a receptor tyrosine kinase which binds to and is activated by several polypeptide ligands including EGF and transforming growth factor α. During mouse development, EGF-R protein has been detected as early as at the 8-cell stage, and later EGF binding activity is present in most of the tissues, peaking around midgestation. The EGF-R is also widely expressed in adult mouse tissues, particularly in the liver, and it has often been found to be amplified and overexpressed in many human tumors. Despite numerous *in vitro* data the function of the EGF-R *in vivo* is poorly understood. Investigations into its role during development and tumorigenesis by overexpressing the gene in transgenic mice have so far been unsuccessful (see also Report 1993).

We have used gene targeting in ES cells to generate mice lacking functional EGF-R. Following homologous recombination, parts of the first exon including the translation initiation site and the leader peptide were replaced in-frame by an *E.coli* lac Z gene. Three targeted ES cell clones were used to generate chimaeric mice all of which transmitted the mutated allele to their offspring. We used β-galactosidase activity to redefine the precise stage and tissue-specific expression pattern of the EGF-R during development. Heterozygous animals show no phenotypic alterations and are fertile, whereas no EGF-R -/- newborns were obtained. Mutant embryos die at mid- to late gestation (**Fig. 6**) where maximal expression of the EGF-R is observed. We are currently analyzing the cause of growth retardation and death observed in EGF-R deficient embryos.

In addition, we have generated ES cells lacking both EGF-R alleles which are viable and not impaired in their growth potential. Interestingly, EGF-R-/- ES cell derivatives can contribute to the development of

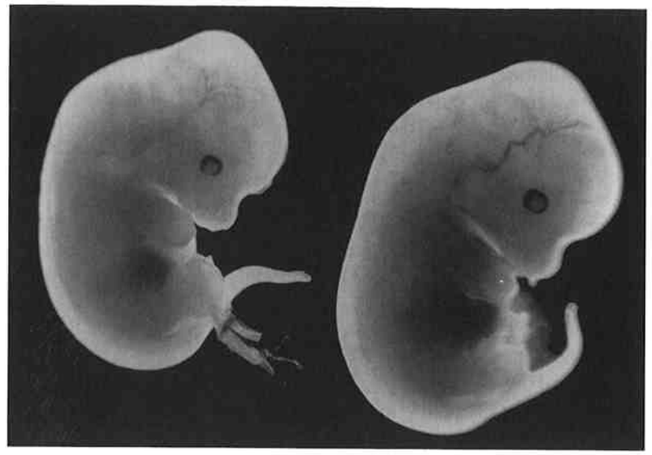


Fig.6: Growth retardation of an EGF-R -/- mouse fetus at day 12.5 of embryonic development.

all organs in adult chimaeric mice but we cannot exclude a cell autonomous defect in a particular cell lineage of the developing organs. We have also isolated EGF-R-/- primary embryonic fibroblasts and established spontaneously immortalized cell lines following the 3T3 protocol. Whereas the proliferation capacity of the primary EGF-R deficient fibroblasts does not seem to be altered, the immortalization process appears to be much slower. We will next investigate how the downstream signaling is affected in these EGF-R mutant fibroblasts.

2. Functional analysis of the *Igf-2/Mpr* gene

Zhao-Qi Wang, Marion Fung in collaboration with Denise Barlow

The mouse *Igf2/Mpr* gene (insulin-like growth factor type 2/mannose 6-phosphate receptor) encodes a lysosome targeting receptor that transfers enzymes and proteins, including the growth factor Igf2, into lysosomes from intra- and extracellular compartments. Both *Igf2* and *Igf2/Mpr* are imprinted, but whereas *Igf2* is expressed exclusively from a paternally-inherited chromosome, *Igf2/Mpr* shows exclusive maternal expression in mid-gestation embryos. To elucidate the function of *Igf2/Mpr* during development and to test whether the inactivation of this gene is responsible for the *Tme* phenotype (see Report Denise Barlow), we disrupted the *Igf2/Mpr* gene in ES cells and in mice. The phenotype from maternal transmission of an inactivated allele and also from homozygous null mutant mice is almost the same. Mice usually die at birth and display increased body weight and morphological alterations reminiscent of the *Tme* mutant. However, a low frequency of mutant mice with a maternally transmitted inactivated allele survive to adulthood, most likely due to reactivated expression of *Igf2/Mpr*. Immunofluorescence labeling studies demonstrated that mutant embryonic fibroblasts were unable to internalize an *Igf2/Mpr* antibody and contained less lysosomal enzymes than wild-type cells. Our studies show that the *Igf2/Mpr* gene regulates embryonic growth and that the receptor functions primarily in regulating *Igf2* levels, since it was possible to rescue the phenotype by the introduction of an *Igf2* null allele.

V. 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 1993). 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.

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Regulation of Protein Serine/Threonine Kinases

Group leader Lisa M. BALLOU
Postdoc Christian KÜHNE (until Oct. 1994)
PhD student Helga EDELMANN
PhD student Claudia PETRITSCH

Introduction

Addition of mitogens to quiescent mammalian cells induces a number of responses that together result in DNA synthesis and cell division. One of the earliest responses is the phosphorylation of ribosomal protein S6. S6 phosphorylation is thought to increase the rate of synthesis of certain proteins, such as ribosomal proteins and protein synthesis elongation factors, that are required for G1 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 well-defined network that includes ras, raf-1 and Mek1 (Pelech, 1993) (**Fig. 1**). By contrast, $p70^{S6k}$ lies on a distinct pathway that does not include MAP kinases (Ballou *et al.*, 1991) (**Fig. 1**). Little is known about the signaling components that function in this second pathway.

$p70^{S6k}$ is the major S6 kinase in fibroblasts and exists as two isoforms of M_r=70,000 and 85,000. The two proteins are identical except that $p85^{S6k}$ has an additional 23 amino acids at the N terminus that act as a nuclear targeting sequence (Reinhard *et al.*, 1994). *In vivo* $^{32}P_i$ labeling and sequencing of phosphopeptides led to the identification of four mitogen-induced phosphorylation sites that are associated with $p70^{S6k}$ activation (Ferrari *et al.*, 1993) (**Fig. 2**). These sites occur within the motif Ser/Thr-Pro and are clustered at the C terminus of the protein. The Ser/Thr-Pro motif is a consensus sequence for phosphorylation by MAP kinases and cyclin-dependent kinases; however, there is no evidence that enzymes in these families activate $p70^{S6k}$. In addition to the four mitogen-responsive phosphorylation sites, $p70^{S6k}$ contains additional phosphates that turn over very slowly and that are also essential for enzyme activity (**Fig. 2**). Rapamycin, an immunosuppressant that arrests the growth of some cell types in G1, induces the dephosphorylation of these novel unmapped sites (Ferrari *et al.*, 1993) and therefore prevents the activation of $p70^{S6k}$ (Chung *et al.*, 1992). The presence of these two groups of phosphates suggests that two or more phosphorylation events are involved in regulating the activity of the enzyme.

The main focus of our research is 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 Lisa M. Ballou

In analogy with the mitogen-induced pathway leading to 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 are using 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, most likely because the phosphatase treatment stripped both the mitogen-induced and the rapamycin-sensitive phosphates from the enzyme. We have now produced large amounts of wild-type and mutant forms of $p70^{S6k}$ in insect cells. The wild-type protein is partially phosphorylated and can be used directly as a substrate to look for enzymes that increase its activity. The ideal substrate to search for the mitogen-activated S6 kinase kinase, however, would be a $p70^{S6k}$ molecule in which the rapamycin-sensitive sites are mutated to

acidic residues. This might mimic phosphate groups and preserve the structure of the enzyme. Such a construct cannot be made until these phosphorylation sites are mapped. A mutant form of p70^{S6k} that has the four known mitogen-stimulated sites mutated to Ala will be used to screen for kinases that phosphorylate the rapamycin-sensitive sites.

In a genetic approach to identify kinases that modify p70^{S6k}, we used two hybrid screening in *S. cerevisiae* 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. We plan to obtain a full-length sequence of this clone and to search the data base to find any clues as to what its function might be. Class II clones also encode the 3' end of an unknown gene but it has some sequence similarity to a known kinase subunit. We will make antibodies to the putative protein fragment and test whether they precipitate an enzyme that phosphorylates p70^{S6k}. We will also obtain a full-length clone to confirm the identity of this protein. 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. We have found that this kinase phosphorylates p70^{S6k} at sites distinct from the mitogen-activated sites. We plan to further map these phosphorylation sites and to test whether they are related to the rapamycin-sensitive sites. We will concentrate on characterizing the class IV and II gene products and determine whether they are involved in regulating p70^{S6k} function.

In this first two hybrid screen one and possibly two kinases were obtained, but substrates such as S6 and CREM (de Groot *et al.*, 1994) were not isolated. This is not surprising, as the p70^{S6k} expressed in yeast is inactive and therefore may not bind strongly to its substrates *in vivo*. When the rapamycin-sensitive phosphorylation sites are mapped, a second screen could be done using a form of p70^{S6k} in which all the known phosphorylation sites are mutated to acidic residues. This might allow the isolation of a different set of clones which could include substrates, S6 kinase phosphatases or other negative regulators.

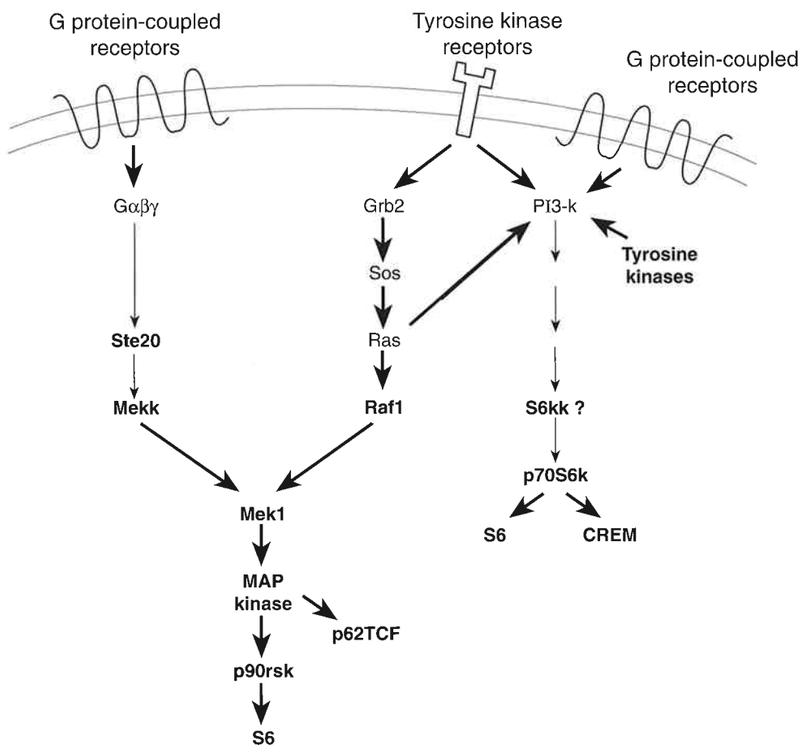

Fig. 1: Signal transduction pathways leading to the activation of p90^{rsk} and p70^{S6k}.

Upstream regulators of p70^{S6k}

Claudia Petritsch (in collaboration with Rüdiger Woscholski and Peter Parker, 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 theophylline or related compounds reversibly blocks the phosphorylation and activation of p70^{S6k} but has no effect on p90^{rsk} or MAP kinases (Petritsch *et al.*, in prep.). Theophylline also causes a decrease in S6 kinase activity when it is added after the mitogen. These results suggested that these compounds either activate a phosphatase that negatively regulates the pathway or inhibit a kinase that positively regulates the pathway. Theophylline is best known as a non-specific phosphodiesterase inhibitor that elevates cAMP and cGMP levels in cells. Our initial hypothesis to explain the effect of theophylline was that high cyclic nucleotide levels activate a cyclic nucleotide-dependent kinase which phosphorylates an enzyme in the p70^{S6k} cascade to inhibit it. However, experiments using cyclic nucleotide analogs, specific phosphodiesterase inhibitors and a cell line deficient in cAMP-dependent protein kinase indicated that cyclic nucleotides and cyclic nucleotide-dependent kinases probably do not play a role in the inhibition of S6 kinase by theophylline (Petritsch *et al.*, in prep.).

Rapamycin, like theophylline, also blocks the activation of p70^{S6k}. Recently it was shown that rapamycin suppresses the growth of yeast by interacting with two gene products encoded by TOR1 and TOR2 (Kunz *et al.*, 1993). These proteins exhibit significant homology to the catalytic subunit of mammalian phosphatidylinositol (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 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 nanomolar concentrations of these compounds irreversibly blocked the activation of p70^{S6k} induced by a variety of external stimuli (Petritsch *et al.*, subm.). By contrast, activation of p90^{rsk} and MAP kinases was not affected.

Although the results above support a model in which PI 3-kinase acts upstream of p70^{S6k} in a mitogenic signaling cascade, this conclusion has recently been questioned (Ming *et al.*, 1994). We therefore are extending this work in two directions. First, COS cells are being transfected with wild-type and mutant forms of PI 3-kinase to see whether p70^{S6k} activity can be manipulated *in vivo*. Second, we are developing a cell-free activation system in which purified inositol phospholipids or PI 3-kinase (wild-type and mutant) can be added to extracts containing inactive p70^{S6k}. This system is also being used to test whether certain protein kinase C isoforms which have been reported to be activated by phospholipids can initiate the p70^{S6k} pathway.

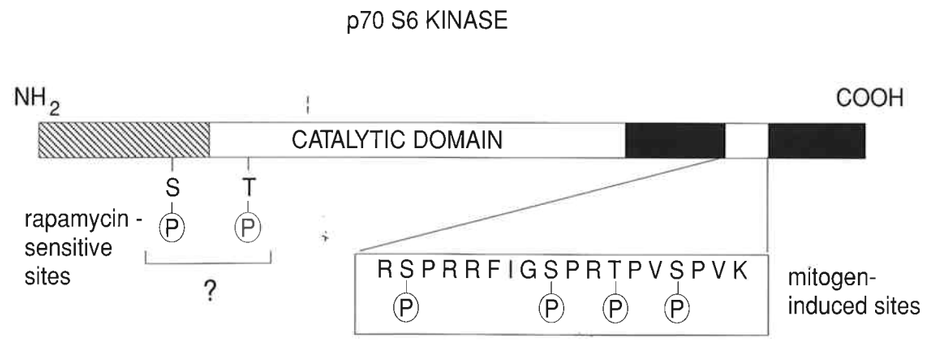

Fig. 2: Structure of p70^{S6k}. Location and number of the rapamycin sites has not been determined.

Cell cycle regulation of p70^{S6k}

Helga Edelmann

Although it is well established that p70^{S6k} is activated as much as 50-fold during the G0-G1 transition, it is not yet clear whether this activity is essential for cell cycle progression. On one hand, microinjection of antibodies that inhibit p70^{S6k} into fibroblasts at any time during G1 blocked entry into S phase (Lane *et al.*, 1993). On the other hand, rapamycin prevented T cells from entering the cell cycle but did not arrest cells that were already in G1, even though the kinase was inhibited (Terada *et al.*, 1993). These results suggest that the function of the kinase might be different during G0-G1 as compared with G1 in cycling cells, and that this difference might be cell type-specific.

To address these questions and to determine whether p70^{S6k} is activated at other times during the mitotic cell cycle, the cell cycle regulation of the enzyme was examined and its characteristics were compared with those of MAP kinases. Fibroblasts were synchronized in G0 by serum starvation. Within 20 min after addition of mitogens, both p70^{S6k} and MAP kinases were phosphorylated and activated (Edelmann *et al.*, in prep.). p70^{S6k} remained highly active throughout G1; during S, G2 and M the activity decreased constantly. In contrast, MAP kinases were active only during early G1. This regulation appeared to be mediated mainly through the phosphorylation state of the enzymes, as protein levels did not change significantly.

To determine if p70^{S6k} and MAP kinases are activated in cycling cells in G1, cells were synchronized in M phase with nocodazole. After washing away the drug, p70^{S6k} became active as cells entered G1. MAP kinases, on the other hand, were activated during telophase and remained active in early G1. We have now produced cell lines stably overexpressing wild-type and mutant forms of p70^{S6k} and are determining whether their cell cycle parameters are altered. A preliminary analysis of the regulation of one mutant form of p70^{S6k} has suggested that an inhibitor of the enzyme might appear at certain stages of the cell cycle (Edelmann *et al.*, in prep.). This possibility is being investigated by doing extract mixing experiments and co-immunoprecipitations from labeled cells.

In addition to changes in activity, immunofluorescence studies showed that p70^{S6k} changes its intracellular localization during the cell cycle (Edelmann *et al.*, in prep.). A deletion analysis may indicate which region of the p70^{S6k} molecule mediates its transport into the nucleus. In addition, we will explore the possibility that the DNA-binding protein isolated in the yeast two hybrid screen (see above) binds to p70^{S6k} or p85^{S6k} in the nucleus. Finally, we will overexpress wild-type and mutant forms of p70^{S6k} fused to a strong nuclear localization sequence to determine whether this affects cell cycle progression.

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Mammalian Developmental Genetics

Mouse Mutants as Tools to Isolate Developmental Control Genes

Group leader	Denise P. BARLOW
Postdoc	Giovanna BRAIDOTTI
Postdoc	Heinz HIMMELBAUER
Postdoc	Björn NEUMANN
PhD student	Pavel KUBICKA (until Sept. 94)
PhD student	Norbert SCHWEIFER
PhD student	Oskar SMRZKA
PhD student	Anton WUTZ
Diploma student	Walter LERCHNER (since Oct. 94)
Technical assistant	Malvina KUPINSKI (since Dec. 94)

Introduction

Growth of the mammalian embryo depends on implantation into the maternal uterus and the formation of a placenta that permits maternal-fetal interactions throughout 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 so that the fetus remains small enough to exit from the uterus. 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 on the embryo. The primary goal of our research is to clone and characterize genes involved in these processes. We are attempting this using the novel strategy of isolating embryonically-expressed genes that are subject to gametic imprinting.

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; Ohlsson *et al.*, 1994) can, however, restrict expression to one parental chromosome. In the mammal, imprinted genes primarily act in embryogenesis and while there no clear understanding why this is so, it is becoming evident that they play a fundamental role in growth control. Of the 12 known mammalian imprinted genes, 6 have been shown to play a role in embryonic growth or tumor growth control (see Table below), the remainder have not yet been analyzed.

gene	*expression*	*function*
Igf2/Mpr	maternal	growth and lysosome function
Igf2	paternal	growth control
H19	maternal	putative tumor suppresser
Mash2	maternal	placental differentiation
Insulin2	paternal	growth and metabolism
Wt1	paternal	tumor suppresser (Wilms' tumor gene)
Xist	paternal	chromosome inactivation
ZN127	paternal	unknown
Snrpn	paternal	unknown
Sp2	paternal	unknown
Par1	paternal	unknown
Par2	paternal	unknown

Tab. 1: The function of imprinted genes

Q1: What is the function of the imprinted Igf2/Mpr gene

Our earlier work (Barlow *et al.*, 1991) demonstrated that the insulin-like growth factor type 2 receptor, also known as the cation-independent mannose-6-phosphate receptor (*Igf2/Mpr*), is imprinted and maternally-expressed in the embryo. Since this imprinted gene was known from biochemical studies to primarily play a role in lysosomal targeting, we generated mice (in collaboration with the laboratory of Erwin Wagner) lacking *Igf2/Mpr,* to define its function in embryogenesis. Our results suggest that *Igf2/Mpr* plays a dual role in mammals, acting in the adult to maintain lysosome activity and in the embryo to inhibit growth (Wang *et al.*, 1994). Figure 1 shows that mice lacking *Igf2/Mpr* are 30% larger at birth than littermates.

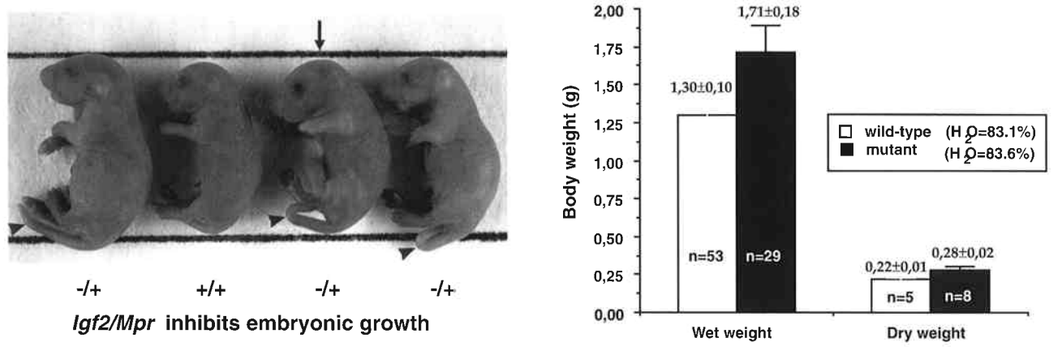

Fig. 1: *Igf2/Mpr* inhibits embryonic growth

Q2: What is the molecular basis of imprinting Igf2/Mpr?

A model has been proposed whereby gametic imprinting is viewed as resulting from several consecutive events (see **Fig. 2**). First, modification of an imprinting box by an imprinting signal, in one of the haploid gametes. Second, maintenance of the imprint on one parental allele in diploid cells. Third, "reading" of the imprint by transcription factors that produce monoallelic expression. Finally, erasure of the imprint in the germ cells of the diploid organism.

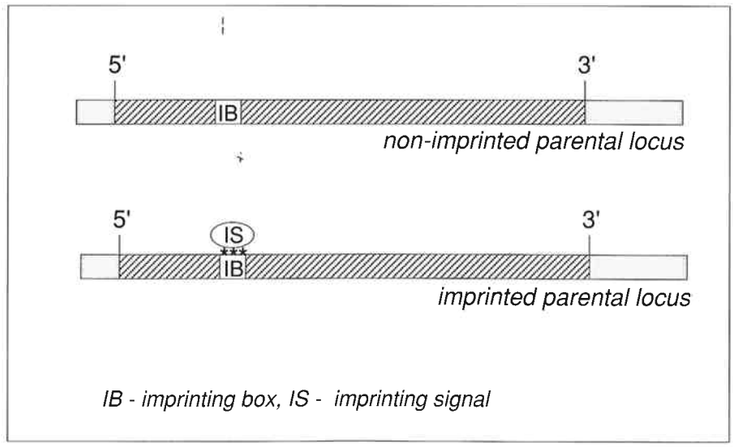

Fig. 2: The nature of the imprint

Previous work from our laboratory identified an intronic element (named Region 2) in *Igf2/Mpr* (Stöger *et al.*, 1993). This element inherited a methylation mark from the female gamete, that remained on the maternal *i.e., expressed* chromosome in diploid cells. We have suggested that methylation constitutes the imprinting signal and that region 2 is the imprinting box for *Igf2/Mpr*, moreover we propose that the methylation imprint causes maternal-specific expression. Currently we are testing the hypothesis that methylation of region 2 inhibits the binding of a repressor protein, thus allowing maternal-specific expression of this gene. Reporter constructs are being investigated that place a luciferase gene under the control of the *Igf2r/Mpr* promoter and region 2. Our hypothesis, that methylation is needed for monoallelic expression at *Igf2/Mpr* has been supported by the demonstration that this gene is silenced in embryos that lack DNA methylation (Li *et al.*, 1993). The hypothesis that region 2 functions as an "imprinting box" for the *Igf2/Mpr* gene is being tested in transgenic mice containing YAC clones encompassing the entire gene of 90 kb, and also by smaller reporter constructs. Transgenic mice have been generated and are undergoing preliminary analysis.

Q3: Common characteristics of imprinted genes

Our identification of a candidate imprinting box in the mouse *Igf2/Mpr* gene prompted an analysis of other sequenced, imprinted genes, to ascertain what elements these genes have in common. The results are shown in **Tab. 2** and show that while the current set of imprinted endogenous genes and transgenes, lack direct sequence homology, they do have many characteristics in common (Neumann *et al.*, 1995).

Gene	CG rich	direct repeats	repeat size	mono-parental methylation
Igf2/Mpr	+	+	25-75	+
Igf2	+	+	42	+
U2afbp-rs	+	+	25-46	+
SNRPN	+	+	15-24	+
TGA transgene	+	+	24-116	+
IAP	+	+	30-115	+

Tab. 2: Common characteristics of imprinted genes

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 that contain direct repeats.

Q4: 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 (Smrzka et al., submitted). 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 *IGF2/MPR* gene) in tumors such as human breast cancer might be associated with abnormalities of imprinting.

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Virus Entry Mechanisms and Gene Delivery

Group leader	Matthew COTTEN
Technical coordinator	Mediyha SALTIK
Postdoc	Susanna CHIOCCA
PhD student	Adam BAKER
PhD student	Heike LEHRMANN (since Oct. 94)
Diploma student	Birgit PANZENBÖCK (since Nov. 94)

Research Topics

We are studying the process and the consequences of adenovirus entry into eukaryotic cells. 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 group 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. We have found that the entry of adenovirus triggers a series of important host responses including the activation of an inflammatory transcription factor (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. Lastly, the development of an efficient gene transfer system has provided the basis for a clinical application of a tumor vaccine that was initiated by Bender in December of 1994 (see also the reports of Max Birnstiel and Ernst Wagner).

Several years have passed since transferrinfection was developed as a method of using receptor-mediated endocytosis to deliver DNA (reviewed in Wagner *et al.*, 1994; Cotten and Wagner, 1993; Cotten *et al.*, 1993a). The currently used transferrinfection system (developed with the groups of Max Birnstiel and Ernst Wagner) is briefly described in **Fig. 1** (see the Fig. 1 legend for details). A crucial component of the system is a psoralen-inactivated adenovirus particle (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. Because we do not understand how the adenovirus particle performs these functions we are studying the process. 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 :

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

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

3.The chicken adenovirus CELO possesses anti-apoptotic genes.

4.The adenovirus protease is directly required during virus entry.

5.Adenovirus entry in the presence of LPS is toxic to primary human cells.

A summary of the consequences of adenovirus entry can be found in **Fig. 2**.

Over the past year, the work of our research group has led to several patent applications. These applications concern:

1. A method of treating cells with agents that block the toxicity generated by LPS during adenovirus entry,

2. Methods of blocking apoptosis or inflammation induced by DNA delivery,

3. The identification and use of a novel anti-apoptotic gene from CELO virus.

Adenovirus capsid binding activates an inflammatory response.

Adam Baker (with Mediyha Saltik, Birgit Panzenböck and Heike Lehrmann)

It has long been known that adenovirus infection can activate an interferon response and more recently that adenovirus infection can activate IL-6 and IL-8 secretion by infected cells. It was tacitly assumed that some form of early virus gene expression was responsible for activating this inflammatory response. Based on this (perhaps) false assumption, other gene therapy groups are generating adenovirus vectors lacking additional genes with the hope that decreasing viral gene expression will decrease the inflammatory response. Using psoralen-inactivated adenovirus particles which are transcriptionally inert, we find that inactive adenovirus stimulates NF-κB and NF-IL6 dependent promoters similar to active adenovirus. Thus, the entry of the virus capsid itself (without virus gene expression) is sufficient to activate these promoters. Fortunately, there are many methods of blunting this inflammatory response and our experiments have demonstrated that anti-inflammatory drugs can enhance long term gene delivery.

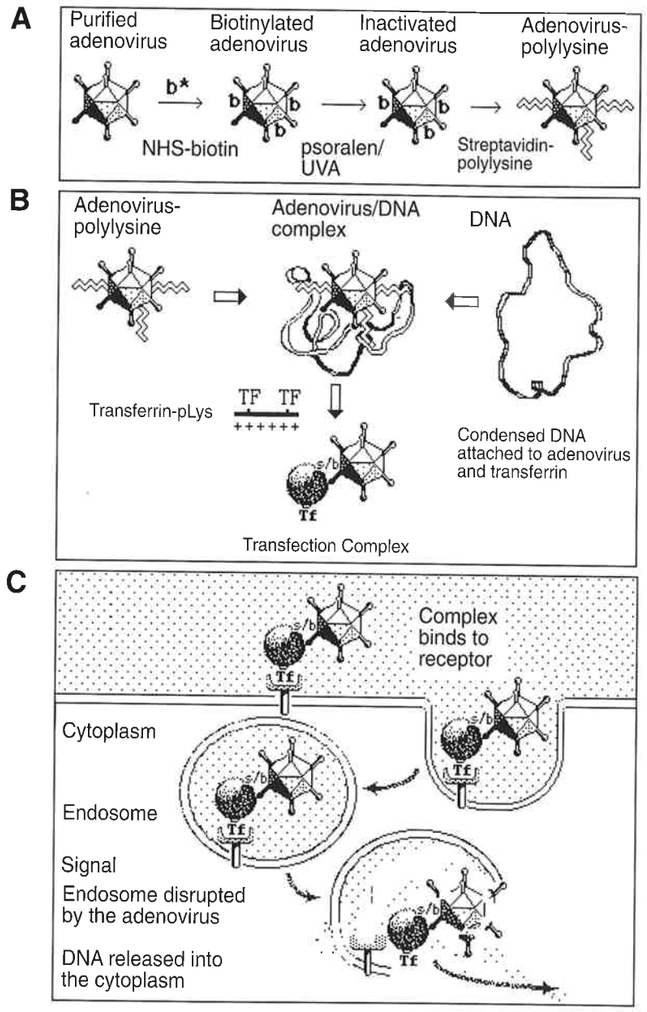

Fig. 1A: Biotinylation and UV inactivation of adenovirus. Exposed lysine residues on the surface of adenovirus particles are modifed with biotin groups. The biotinylated virus is then treated with the DNA intercalator 8-methoxypsoralen and irradiated with 360 nm UV light to destroy the viral DNA. DNA binding polylysine is then attached to the exterior of the virus using streptavidin-polylysine.

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.

Apoptosis Introduction

It is becoming apparent that apoptosis is a frequent eukaryotic host cell response to DNA virus entry. This is clearly of benefit to a multicellular organism: a single infected cell dies before the invading virus can replicate and infect additional cells. This apoptosis is thought to be triggered either directly, by the viral gene expression or indirectly, by immune system intervention such as via TNF. However, many viruses possess genes that block the apoptotic response and allow virus replication. The *deg* phenotype (an early description of apoptosis; D'Halluin *et al.*, 1979; Ezoe *et al.*, 1981) was found to be due to mutations in the E1B 19K protein (Pilder *et al.*, 1984; Takemori *et al.*, 1984) and it is now clear that apoptosis can be generated by expression of an E1A growth signal in the absence of E1B (Rao *et al.*, 1992). It has also been demonstrated that the E1B 19K protein can function as a potent analogue of the apoptosis-blocking mammalian gene Bcl-2 (Rao *et al.*, 1992).

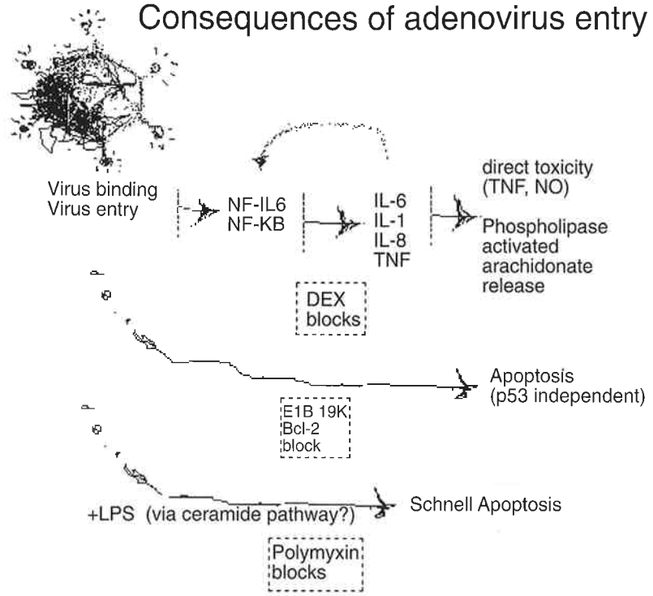

Fig. 2.: The consequences of adenovirus entry. The binding and entry of adenovirus particles can activate an apoptotic response (blocked by E1B 19K) and an inflammatory response (blocked by glucocorticoids such as dexamethasone). In the presence of lipopolysaccharide (LPS, a common contaminant of DNA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

Gene expression following transfection is limited by apoptosis

Adam Baker

We have found that cell death is triggered by the entry of the adenovirus, even in the absence of viral gene expression. Co-introduction of an active adenovirus E1 region provided some relief from the apoptosis, with further experiments using plasmids bearing individual E1 genes identifying 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 (Rao *et al.*, 1992; Debbas and White, 1993). Expression of either protein can block apoptosis triggered by a number of signals. Consistent with this apoptotic phenotype, co-introduction of the Bcl-2 gene into cells could also block the toxicity.

The E1B 55K gene product functions to bind and sequester p53. However, unlike the protection seen with the E1B 19K product, co-transfection with E1B 55K expression plasmid does not protect cells suggesting that the apoptotic event is independent of p53. Co-transfection with plasmids expressing either the dominant negative, val 135 p53 or the p53 sequestering protein mdm2 failed to replace the E1B 19k function, further demonstrating that the apoptotic pathway activated by transferrinfection does not involve p53.

Eventually, however, even the E1B-19K or Bcl-2 expressing cells begin to lose gene activity at later times (2-3 weeks) after transfection. A second response related to the inflammatory response caused by adenovirus entry appears to compromise the health of the transfected culture and the inclusion of anti-inflammatory agents enhances long term gene expression.

Identification of an anti-apoptotic gene in CELO virus.

Susanna Chiocca (with Adam Baker, Mediyha Saltik, Robert Kurzbauer and Gotthold Schaffner)

We have demonstrated that the decline in cell viability induced by adenovirus-augmented transferrinfection in primary human fibroblasts can be prevented by expression of the anti-apoptotic genes E1B 19K or Bcl-2 (see Adam Baker abstract above). This forms the basis of a screen for anti-apoptotic genes (**Fig. 3**). We simply co-transfect a luciferase expression plasmid with a test gene and look for an enhancement in

long-term gene expression. We have previously demonstrated that gene delivery with the avian adenovirus type 1 (CELO virus) can produce stably transduced cells at a very high frequency (Cotten *et al.*, 1993b). In light of our observation that transferrinfection limits the viability of the transduced cell, we are testing the hypothesis that the CELO virus expresses anti-apoptotic, E1B 19K-like genes. To do this, the entire CELO virus genome was subcloned onto bacterial plasmids and individual plasmids bearing CELO virus sequences were co-transfected with a marker gene into primary fibroblasts. We have found enhancement of long-term gene expression with plasmids bearing two portions of the CELO genome. One identified region (GAM1) has a single large open reading frame with no homologies to any of the published anti-apoptotic genes. The location of the gene suggests that it may be part of the CELO E3 unit and the anti-apoptotic function of this gene is consistent with known functions of the mammalian E3 in the inhibition of TNF signaling.

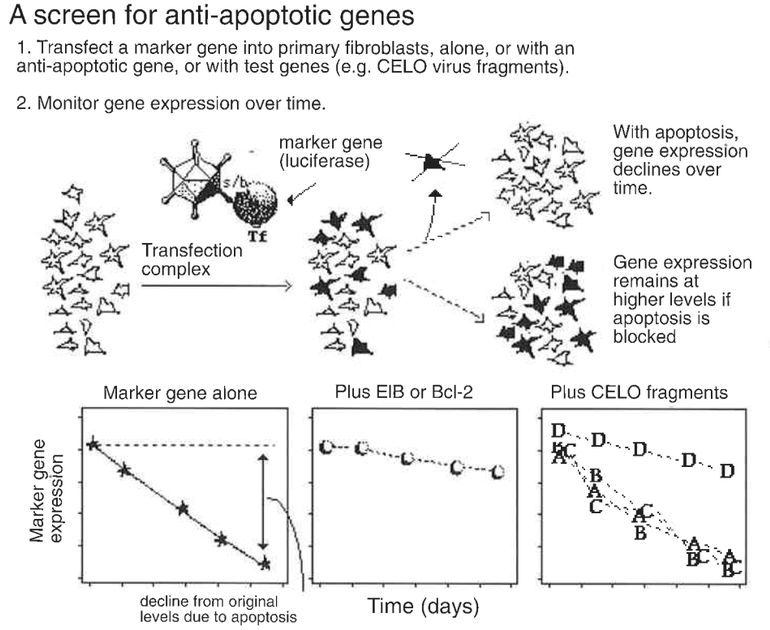

Fig. 3: A screen for anti-apoptotic genes.

Toxicity to primary cells in the presence of LPS and adenovirus.

Matt Cotten (with Adam Baker, Mediyha Saltik (IMP) Ernst Wagner (IMP and Bender) and Michael Buschle (Bender))

LPS (lipopolysaccharide, endotoxin) is a major component of the gram-negative bacterial cell wall and is released from E. coli when the bacteria are lysed to prepare plasmid DNA. The chemical properties of LPS allow the molecule to behave like DNA on both anion exchange chromatographic resins and on CsCl gradients. Thus, biologically significant quantities of LPS are commonly found as contaminants of plasmid DNA preparations. We have found that these quantities of LPS can generate a toxicity to primary cells (primary human skin fibroblasts, primary human melanoma cells) in the presence of adenovirus particles (Cotten *et al.*, 1994b). Simple and effective methods of removing the contaminating LPS using either a polymyxin B resin or Triton X-114 extraction have been developed and purification of DNA samples to remove LPS eliminates the toxicity to primary cells (Cotten *et al.*, 1994b).

The DNA source of LPS can be readily removed. However, LPS can also be encountered in gene therapy environments *in vivo*, such as in the cystic fibrosis lung, where it is not possible to remove the toxin. We have identified small molecular compounds that can be used to neutralize the toxic effects of LPS *in situ,* thus allowing gene delivery in the presence of LPS.

Adenovirus protease is required for virus entry

Matt Cotten (with Mediyha Saltik and Joseph Weber, Université de Sherbrooke, Canada)

During adenovirus assembly, adenovirus type 2 and 5 generate capsid shells that are deficient in their DNA content. These empty shells possess the major exterior capsid proteins, lack the full content of viral DNA and can be purified from full adenovirus particles due to their differences in buoyant density. These empty particles should be an ideal reagent for augmenting DNA or protein delivery because of their low content of viral nucleic acid and correspondingly low viral gene expression. However, we have found that these immature virus particles are deficient for augmenting gene delivery (Cotten and Weber, 1995). Apart from the lowered DNA content of the empty particles, they possess other significant differences from full mature virus particles in the maturation status of the capsid proteins as well as the absence of full viral protease activity. The entry of DNA into the adenovirus capsid activates a viral-encoded protease, the L3 23K protease, to cleave 6 capsid proteins at defined sites. Empty capsids have not yet undergone this proteolytic processing (see **Fig. 4** for a summary of adenovirus maturation). Using an adenovirus strain that possesses a temperature sensitive protease (Ad2 ts1; Weber, 1976) we have found that virus grown at the permissive temperature (32 °C) is fully active for DNA delivery and for pH-dependent membrane disruption. In sharp contrast, ts1 adenovirus grown at the non-permissive temperature (39 °C) is defective in these two functions. The temperature sensitive mutant ts4 is defective for DNA entry and produces predominantly empty (DNA deficient) particles at 39 °C. Due to the lack of DNA entry, ts4 does not activate the protease when grown at 39 °C and thus produces only empty, immature particles. We find that this mutant also is defective for DNA delivery.

An additional function of the protease was revealed by experiments using inhibitors of the protease (e.g. copper chloride). Mature, entry-competent virus capsids lose their ability to enhance gene delivery and become non-infectious after treatment with 100-300 μM copper chloride (Cotten and Weber, 1995) suggesting that the viral protease activity is required during the cellular entry process. These experiments indicate that adenovirus entry involves viral enzymatic activity, and models of virus entry must be adapted to include this function in addition to the physical membrane interactions that we had previously thought solely responsible for virus passage through the cell membrane.

Adaptation of the Adenovirus augmented receptor-mediated endocytosis for the delivery of Yeast Artificial Chromosomes (YACs) into eukaryotic cells.

Adam Baker

A YAC is a **y**east **a**rtificial **c**hromosome (Burke *et al.* 1987) which can be used for the isolation and propagation of exogenous DNA segments up to a megabase in length allowing most mammalian genes or gene clusters to be cloned into *Saccharomyces cerevisiae* as a single DNA entity. YACs are very powerful tools for the characterization of complex mammalian genomes, allowing the isolation and study of large gene and gene clusters.

For full utilization of their potential, YACs need to be transferred intact into mammalian cells where proper functional analysis of the DNA can take place. Transfer of YACs using fusion of mammalian cells with yeast spheroblasts has allowedYACs of up to 670kb to be introduced into mammalian cells (Jakobovits e*t al*, 1993). However this method introduces a large quantity of yeast genomic DNA into the cell along with the YAC and is very sensitive to the type of cell used. Microinjection of purified YAC DNA is the only other way that very large YACs have been introduced into mammalian cells (Gnirke *et al*, 1993). Alternate methods of YAC transfer using cationic lipids, calcium phosphate co-precipitation, and electroporation have, to date, only been successful in delivering YACs of up to 150kb. In addition, a major problem that plagues all methods (apart from microinjection) is the very low transformation rate; the maximum rate being about 1 in 10^5 cells.

We are adapting the transferrinfection system for the delivery of YACs into mammalian cells. Adenovirus augmented receptor-mediated endocytosis has been shown to be a useful method for introducing DNA of up to 48 kb into eukaryotic cells (Cotten e*t al.*, 1992) and the upper limit of this system has not yet been identified. We are developing a library of YACs covering a range of sizes. These YACs contain the standard YAC markers, as well as a bacterial aminoglycoside phosphotransferase gene (neo) under mammalian transcriptional control which serves as a selection marker in mammalian cells and a β-Galactosidase reporter gene for rapid analysis of transfection success. We hope to use what we have learned about DNA condensation (Wagner *et al.*, 1991) to generate an effective method of YAC transfer.

Adenovirus maturation

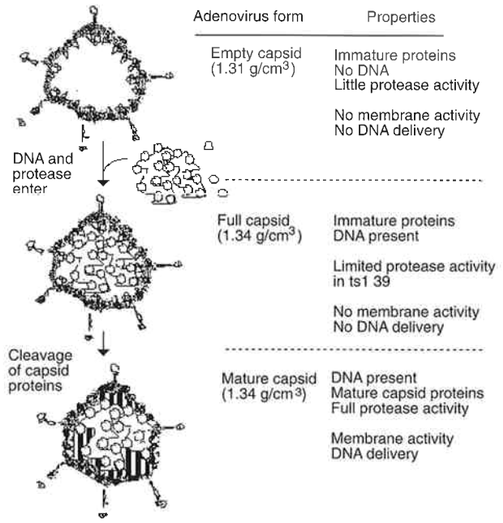

Fig. 4: Adenovirus maturation. Three stages of adenovirus maturation are described. The adenovirus capsid forms as premature shell which has a light density (1.32 g/cm³) due to the absence of DNA. The shell is then filled with DNA (density is now 1.34 g/cm³) followed by activation of the viral protease and processing of six of the viral capsid proteins to generate the mature, entry-competent virus.

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Molecular Dissection of Multistage Tumorigenesis in Transgenic Mice

Group leader Gerhard CHRISTOFORI (since March 94)
Postdoc Gabor LAMM (since Sept. 94)
PhD student Anne-Karina PERL (since Aug. 94)
Technical assistant Susanne LUEF (since April 94)

Introduction

During development of many tumors, distinctive stages can be recognized: normal unaffected tissue, hyperplasia with a high incidence of proliferating cells, induction of tumor angiogenesis with the new growth of capillaries, solid tumors (neoplasia), and finally metastasis. The molecular analysis of the genetic and epigenetic events involved in multistage tumorigenesis is often hampered by the unavailability of tissue specimens from the different stages. For this reason, the genetic reproducibility and the accessibility of tissue specimens have made transgenic mice a valuable tool to study the molecular events that are involved in the stepwise progression to the tumor phenotype (Hanahan, 1989; Christofori and Hanahan, 1994).

In one transgenic model of tumorigenesis, the rat insulin gene regulatory region (RIP1) has been used to target expression of Simian Virus-40 large T-antigen (Tag) to the β cells of the pancreatic islets, of which the RIP1Tag2 line has been the prototype (Hanahan, 1985). These mice develop tumors in the β 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 β 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 (Teitelman *et al.*, 1988). 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 chemoattractive and mitogenic for endothelial cells (Folkman *et al.*, 1989). 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 are involved in the induction of tumor cell hyperproliferation, the cause of neovascularization during tumor development (tumor angiogenesis), and factors that determine the tumorigenicity of tumor cells in RIP1Tag2 transgenic mice. We employ these transgenic mouse models to prove causal connections between the expression of a gene and tumor progression *in vivo*. For example, genetic complementation of the RIP1Tag2 transgenic 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 β cell tumorigenesis in particular and in cancer development in general.

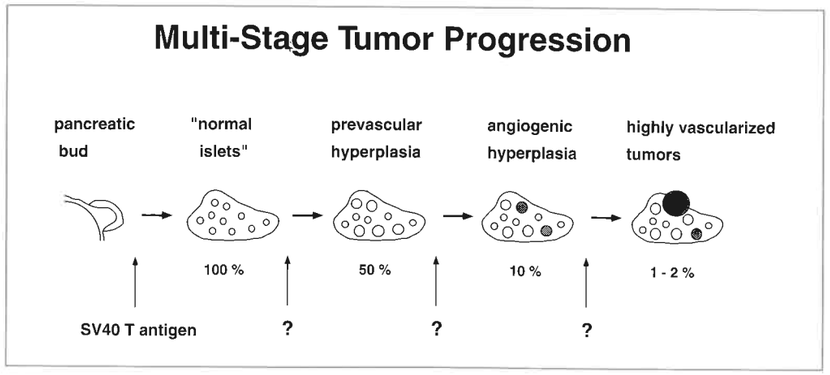

Fig. 1: Schematic representation of multistage tumor progression in RIP1Tag transgenic mice.

I. The Functional Role of IGF-II in β Cell Tumorigenesis

Gabor Lamm and Gerhard Christofori in collaboration with Paul Naik and Douglas Hanahan (UCSF)

The switch from non-proliferating cells to proliferating tumor cells, i.e. the induction of hyperplasia, is a major focus of the laboratory. In most cancers diffusible growth factors are produced by the tumor cells thereby stimulating tumor cell proliferation in an autocrine or paracrine fashion. Our molecular analysis of tumor progression in the RIP1Tag2 transgenic mouse model revealed that activation of insulin-like growth factor (IGF-II) coincided with the switch to the hyperproliferative stage during β cell tumorigenesis. When RIP1Tag2 mice were bred to mice carrying a targeted disruption of the IGF-II gene so as to produce transgenic mice lacking a functional IGF-II allele, tumorigenesis was dramatically impaired (Christofori *et al.*, 1994; Naik *et al.*, 1994). Compared to wild-type IGF-II mice, much smaller tumors of lower grade malignancy developed (**Fig. 2**). While the mitotic index was equally high in the tumors of both genotypes, the incidence of apoptotic cell bodies was 5-fold higher in the tumors of IGF-II-deficient mice suggesting that tumor cell apoptosis is responsible for the differences in tumor volume (**Fig. 3**).

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. In some of the cell lines that have been derived from these cancers growth regulation via IGF-II binding to the IGF-1 receptor has been established (see references in Christofori and Hanahan, 1994).

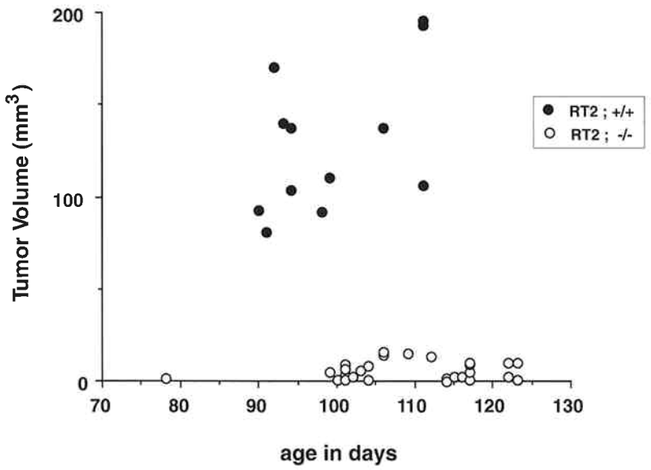

Fig. 2: Comparison of tumor volume between IGF-II proficient and IGF-II null RIP1Tag2 transgenic mice. A histogram shows the large reduction of in tumor volume at autopsy in a series of individual RT2;IGF-II $^{-/-}$ mice compared to RT2;IGF-II $^{+/+}$ control mice.

I.a. Tumor Cell Proliferation versus Tumor Cell Apoptosis

Our recent studies suggest that IGF-II acts as a survival factor for the tumor cells in order to escape programmed cell death. These experiments also reveal that β tumor cell lines that are deficient in IGF-II expression rapidly undergo apoptosis when grown in low serum, whereas wild-type β tumor cell lines do not exhibit a significant incidence of apoptosis when grown under these conditions. We employ this system to functionally characterize genes that are known to play a role in the regulation of apoptosis, such as bcl-2, bcl-x, bax, Mcl-1, ICE, Ich-1/nedd-2, and others (see references in Wyllie, 1994, and Williams and Smith, 1993). Furthermore, we will attempt to identify novel genes that are responsible for the balance between β tumor cell proliferation and β tumor cell death. We will analyze the expression pattern of these genes during tumorigenesis in wild-type and IGF-II-deficient RIP1Tag2 transgenic mice. 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 β cell tumor development. From these experiments we hope to learn about the principles that govern tumor cell survival or death *in vivo*.

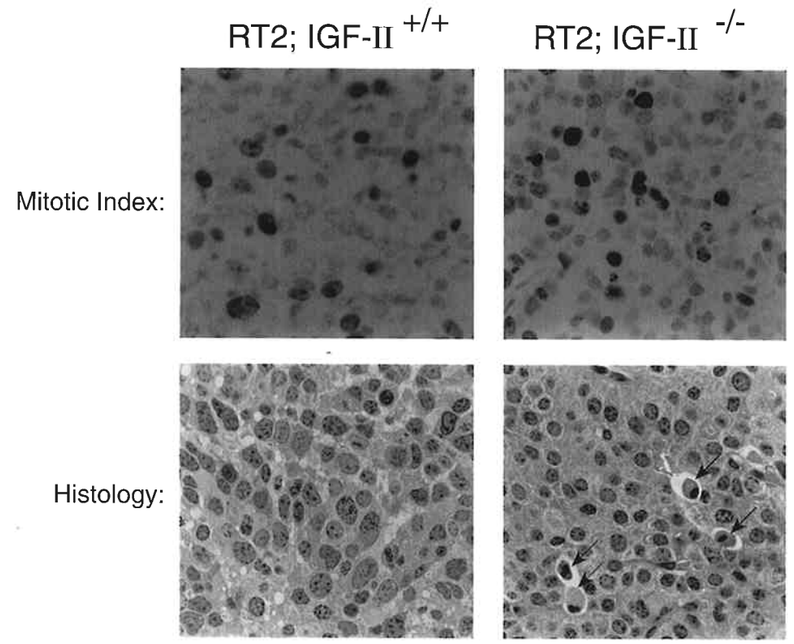

Fig. 3: Comparison of proliferation index and tumor cell apoptosis between IGF-II proficient and IGF-II null RIP1Tag2 transgenic mice.
Mitotic Index: Immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation in tumors derived from RIP1Tag2;IGF-II$^{+/+}$ and RIP1Tag2;IGF-II$^{-/-}$ mice, respectively, revealing a similar high proliferation index in both.
Histology: Hematoxylin and eosin stained semi-thin sections of tumors derived from RIP1Tag2;IGF-II$^{+/+}$ and RIP1Tag2;IGF-II$^{-/-}$ mice, respectively, revealing an approximately 5-fold higher incidence of apoptotic cells in the IGF-II null tumors (indicated by arrows).

I.b. IGF-II Upregulation and the Disruption of Genomic Imprinting

SV40 T antigen is expressed early during embryonic development in all β cells in the RIP1Tag2 transgenic mice. Yet, it takes several weeks before upregulation of IGF-II expression, and with it, hyperproliferation of β cells is first detected. To identify the link between T antigen expression and IGF-II upregulation we plan to functionally characterize the promoter region of the IGF-II gene. Wilms tumor gene product (WT1) and p53 have been reported to regulate IGF-II transcription in co-transfection experiments (Drummond *et al.*, 1992; Maheswaran *et al.*, 1993). We are currently intercrossing the RIP1Tag2 transgenic mice with p53-deficient mice to assess whether the complete absence of p53 will accelerate IGF-II upregulation and/or β cell tumor development. In this context, we will also examine a potential role of WT1 and other WT1 family members in the RIP1Tag2 tumor pathway.

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: (1) genomic imprinting is relaxed or disrupted, (2) the repressive imprinting signal is ignored during transcriptional activation, or (3) different promoters are used between embryonic tissues and tumor cells. We plan to distinguish between these possibilities by determining changes in promoter usage and in methylation status of the IGF-II gene during the multiple stages of β cell tumorigenesis.

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

It is now well established that the formation of new blood vessels from pre-existing vessels (angiogenesis) is required for the outgrowth of most, if not all, solid malignancies (Folkman and Shing, 1992). For this reason we are interested in the molecular mechanisms that are responsible for the onset of 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 (**Fig. 4**; Folkman *et al.*, 1989). Biochemical fractionation of medium conditioned by β 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 (aFGF).

VEGF is secreted by normal β cells, and the mRNA for its receptors (flt-1 and flk-1) is present in endothelial cells of normal islets of Langerhans. The expression of VEGF and its receptors does not significantly change during the different stages of tumor progression. These results indicate that in RIP1Tag2 transgenic mice, the onset of angiogenesis does not coincide with a change in VEGF expression.

In a similar manner, aFGF is expressed in the β cells at all stages of tumor development in RIP1Tag2 transgenic mice. However, although acidic FGF does not have a classical signal sequence for secretion, it is nevertheless released into the culture medium by β tumor cell lines. The aFGF found in the conditioned medium of β tumor cell lines exhibits lower heparin-binding affinity and higher molecular weight as compared to normal aFGF. Preliminary characterization of these higher molecular weight forms suggests that aFGF forms dimers and aggregates with another, unidentified factor. The high molecular weight forms of aFGF are found in the cell lysate as well as in the conditioned medium, and we suspect that they might be intermediates in the export of aFGF (Christofori *et al.*, 1995b). We plan to pursue the characterization of these complexes as an approach to elucidate the molecular mechanism of aFGF release.

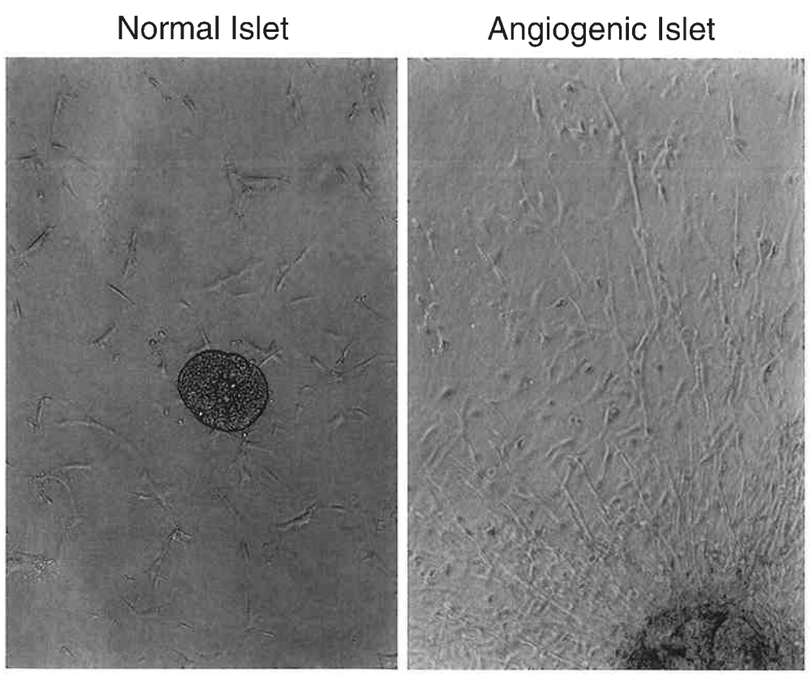

Fig. 4: Co-cultivation of isolated islets from different stages of β cell tumor development with capillary endothelial cells in a three-dimensional collagen gel. Soluble factors that induce proliferation, migration and tube formation of co-cultured capillary endothelial cells are released by angiogenic islets but not by normal or pre-vascular hyperplastic islets.

Export of FGF lacking a signal sequence is not without precedent. A similar export of basic fibroblast growth factor (bFGF) has been reported in cell lines derived from different stages of fibrosarcoma development in transgenic mice carrying the bovine papilloma virus genome (Kandel *et al.*, 1991). In these cell lines bFGF is only exported from cell lines corresponding to the advanced tumor stages, and it is predominantly found in high molecular weight aggregates. More recently, we have detected similar high molecular weight forms of bFGF in the culture medium of several breast carcinoma cell lines. We have initiated experiments to further characterize these forms of bFGF.

The high molecular weight complexes found with both aFGF and bFGF in the cell lines described above resemble each other in their biochemical properties suggesting that both FGFs are released by a common export pathway. In addition to the up-regulation of other angiogenic factors, such as VEGF, and the downregulation of angiogenesis inhibitors, such as thrombospondin, the induction of FGF export may therefore be another general pathway for the onset of tumor angiogenesis. The unravelling of the molecular mechanisms underlying the export of aFGF and bFGF may help to develop therapeutic approaches that will interfere with tumor angiogenesis and, thus, with tumor outgrowth and metastasis.

III. Changes in Cell Adhesion during β Cell Tumor Development

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

III.a. The Role of E-Cadherin in β Cell Tumorigenesis

Molecular analysis of cell adhesion molecule expression during the different stages of β cell tumorigenesis revealed a loss of E-Cadherin expression in large, invasive tumors. Because the loss of E-Cadherin expression has been implicated in the development of invasive epithelial tumors (Vleminckx *et al.*, 1991; Birchmeier and Behrens, 1994), we are particularly interested in determining its role in β cell tumorigenesis in RIP1Tag2 transgenic mice.

All the β tumor cell lines (βTC) tested are highly tumorigenic upon transplantation into immunodeficient mice. In order to up-regulate E-Cadherin expression we have introduced E-Cadherin under the control of the insulin promoter (RIP1E-Cad) into βTC and we are currently evaluating the tumorigenicity of the βTC(E-Cad) cells in immunodeficient mice. Preliminary results show markedly reduced tumorigenicity with the βTC(E-Cad) cell lines as compared to control βTC cell lines.

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

III.b. Switch in N-CAM Isoforms during β Cell Tumorigenesis

Our studies on cell adhesion molecules during β cell tumor development also revealed that the expression of neural cell adhesion molecule (N-CAM) changed in the later stages of tumor development; the 120 kd GPI-linked isoform on the surface of normal β cells is almost completely replaced by the highly polysialylated 140 and 180 kd transmembrane isoforms in β 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 β 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 viable and fertile and, thus, can be employed to produce RIP1Tag2 transgenic mice that lack any functional N-CAM allele. We will examine these mice for phenotypic changes in β cell tumor development. It is possible, however, that the complete absence of N-CAM expression has no direct effect on β cell tumor development since the switch from the 120 kd isoform in normal β cells to the highly polysialylated 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 β 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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Chromatin Domains and Gene Activity during Mouse Development

Group leader Thomas JENUWEIN
Postdoc Götz LAIBLE (since Feb. 94)
PhD student Andrea WOLF (since Oct. 94)
Technical assistant Angelika LEBERSORGER (since April 94)

Introduction

The packaging of DNA into chromosomal domains and the decondensation of higher order chromatin are crucial mechanisms to regulate the activity of key developmental loci in eukaryotes. The "on- or off-state" of these developmentally regulated loci is usually reset during the very early stages of development as a consequence of active restructuring of chromatin. Our research plans focus on the poorly defined mechanisms on how chromatin-dependent alterations in gene expression patterns promote or perturb mammalian cell type specialization and development. In particular, we are using (i) the immunoglobulin heavy chain (μ) enhancer and murine lymphoid differentiation as a model in transgenic mice to dissect the molecular mechanisms of locus control and (ii) are isolating novel mammalian chromatin regulators whose function will ultimately be analyzed in gene targeting experiments.

I. Molecular mechanisms of locus control

Gene expression at heterochromatic sites is severely downregulated as compared to gene expression at euchromatic sites, a phenomenon reflected by position effects in germline transformations. Enhancers and enhancer-like locus control regions (LCRs) have been shown to overcome these position effects, indicating that LCRs govern the correct expression profile of a developmentally regulated gene.

(a) Establishment of an active chromatin domain

Thomas Jenuwein and Götz Laible, in collaboration with Billy Forrester and Rudi Grosschedl (University of California at San Francisco)

The immunoglobulin heavy chain (μ) enhancer region confers high lymphoid-specific transcriptional activity upon the μ gene and heterologous genes in germline transformations, suggesting a role for the μ enhancer region in organizing a chromatin domain. A direct demonstration of this putative function, however, has been experimentally difficult because of the complication to uncouple enhancer-mediated changes in the chromatin structure from those induced by ongoing transcription. Therefore, we started to analyze μ enhancer function in chromatin in the absence of a cis-linked RNA polymerase II promoter. The experimental strategy involves the microinjection into fertilized mouse eggs of minigenes consisting of the binding sites for the prokaryotic T3 and T7 RNA polymerases linked at proximal (T3 promoter) and distal (T7 promoter) positions relative to various fragments from the μ enhancer region. The potential of the μ enhancer region to "open" chromatin for factor access can then be analyzed by measuring the synthesis of T3- and T7-specific run-on transcripts which were generated by incubation of transgenic pre-B cell nuclei with exogenously added T3- or T7 RNA polymerase.

Using this strategy, our data demonstrate that a specific combination of factor binding sites within the μ enhancer core is sufficient to "program" the chromatin structure for localized factor access in the absence of measurable transcription. A/T-rich matrix attachment regions (MARs) flanking the μ enhancer core, however, are required to propagate this localized factor access to distal promoters and to generate DNAse I sensitive chromatin (Forrester *et al.*, 1994; Jenuwein *et al.*, submitted). A model has been proposed, in which the combination of transcription factor binding sites with flanking MARs is important to constitute a functional locus control region to confer long-range alterations in chromatin (Jenuwein *et al.*, 1993; see **Fig. 1**).

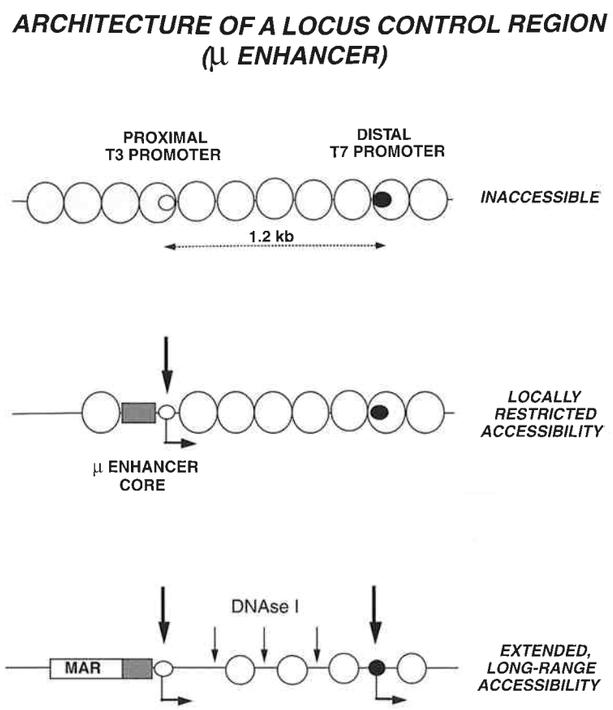

Fig. 1: Architecture of a locus control region (μ enhancer)

(b) Architecture of a locus control region (μ enhancer)

Thomas Jenuwein and Angelika Lebersorger

The requirement for MAR sequences to establish long range factor access could reflect binding to a catalytic surface (the nuclear matrix) or recruitment of accessory proteins. In order to distinguish between these mechanisms, new gene constructs were generated in which the relative orientation and alignment of either the 5'MAR or the 3'MAR with respect to the enhancer core were altered. Preliminary analysis of some of the μT3/T7 transgenes indicated a low basal accessibility of the distal T7 promoter in the absence of an MAR. Addition of either the 5'MAR or the 3'MAR in its correct physiological orientation potentiated distal accessibility by at least one order of magnitude, whereas placement of the 5'MAR in the reverse orientation at the 5' end of the enhancer core resulted in the down-regulation of even the basal accessibility. Since an isolated MAR has been shown to be inactive (Jenuwein *et al.*, 1993), these data suggest that MARs synergize with factor binding sites in the μ enhancer core and, dependent on their orientation, alter the activity of a locus control region.

Using similar μT3/T7 transgenes that contain the μ enhancer region with truncated MAR sequences linked to T3 and T7 promoters at various distal positions, the following additional questions will be addressed: (i) What are the minimal sequence requirements for MAR function? (ii) How far from the μ enhancer region can alterations in chromatin be propagated?

II. Mammalian regulators of the chromatin structure

Our knowledge of mammalian gene products interacting with chromatin to establish and maintain cell type-specific patterns of gene expression is limited. By contrast, the powerful genetics available in yeast and *Drosophila* facilitated the isolation of genes that govern cell type determination through alterations in the chromatin structure. Paradigms include SIR-dependent mating type switching in yeast and *Pc-G*

dependent repression of homeotic selector genes in *Drosophila*. We started to screen mammalian cDNA libraries for homologues of *Drosophila* chromatin regulators and are using a functional test in yeast to identify mammalian cDNAs that complement sir⁻ mutations. Together, these approaches should allow the isolation of mammalian regulators of the chromatin structure whose function will ultimately be analyzed in gene targeting experiments.

(a) Mammalian homologues of Drosophila chromatin regulators

Götz Laible

Genetic analyses in *Drosophila* have facilitated the isolation of several genes whose products appear to be directly involved in mediating cell type determination through chromatin-dependent changes in gene expression. Both activators (e.g. *trithorax* or *trx*-group) and repressors (e.g. *polycomb* or *Pc*-group) of the chromatin structure of homeotic selector genes have been described (see **Fig. 2**). Interestingly, the few known mammalian homologues, like human *HRX* (*trx*-goup) and murine *bmi* (*Pc*-group) 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. Recently, a carboxy-terminal region of sequence similarity has been identified that is shared between a positive (*trx*) and a negative (*e(z)*) *Drosophila* chromatin regulator. Furthermore, this carboxy terminus is also conserved in Su(var)3-9, a dominant suppressor of position effect variegation (PEV). It has been suggested that this region, termed SET domain (Tschiersch *et al.*, 1994), 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.

The screening of a human cDNA library has yielded 38 clones which are putative homologues of *Drosophila* chromatin regulator genes, some of them exhibiting novel restriction patterns. We are currently isolating the full-legth cDNAs from mouse and human cDNA libraries. These full-length cDNAs will be used to raise antibodies from bacterially expressed fusion proteins and to investigate gene function *in vivo* through targeting of the corresponding genomic loci.

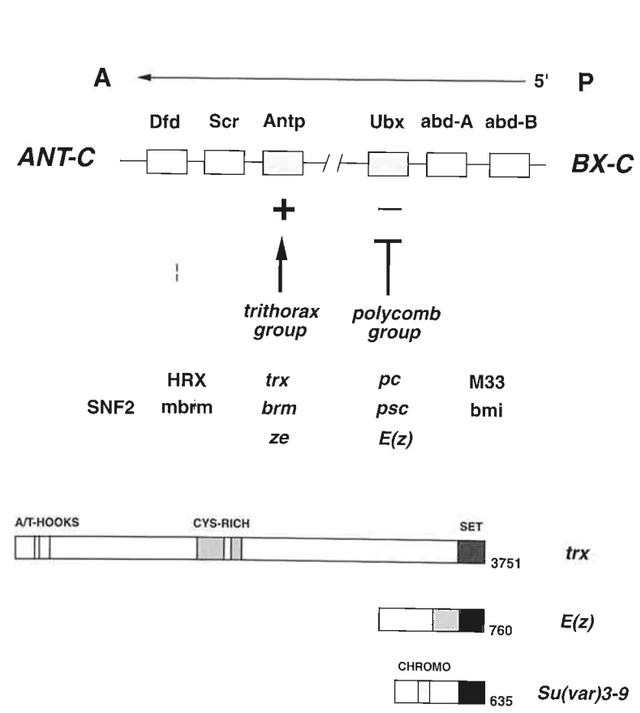

Fig. 2: Drosophila chromatin regulators

(b) Mammalian homologues of yeast SIR genes

Andrea Wolf

Proteins restructuring chromatin do not seem to bind to a specific DNA sequence but rather interact with regulatory proteins already sitting at the target site. A paradigm for this kind of chromatin-dependent gene expression is represented by the yeast mating type locus, MAT. It has been shown that changes in the chromatin structure allow expression at the accessible MAT locus but repress activation at the distant silent loci *HML*a and *HMR***a**. Interestingly, the SIR genes (for **s**ilent **i**nformation **r**egulator) that downregulate the mating type loci are also involved in the repression of gene activity at telomeric positions (Renauld *et al.*, 1993; Moretti *et al.*, 1994), a phenomenon that is reminiscent of PEV in *Drosophila* and X-inactivation in mammals. Because of the specific function of SIR genes in mating type switching in yeast, it is tempting to speculate that mammalian homologues of SIR genes also play key roles in determining chromatin-dependent cell type-specialization in higher eukaryotes.

We are exploiting a double selection system in yeast to complement sir⁻ mutations with an expression cDNA library from human B-cells. First, a telomeric selectable marker gene (ura) has been shown to be derepressed in a sir⁻ background (Renauld *et al.*, 1993). Since the spreading of heterochromatin from the telomere varies with each cell division, the derepression is not complete, and a minor fraction of cells can be counterselected for inactivity of the ura gene (see **Fig. 3**). This variegated phenotype is extremely characteristic of metastable, chromatin-dependent changes in gene expression. Second, sir⁻ mutations inactivate the ability of yeast cells to switch cell types, because all three mating type loci are expressed simultaneously. Therefore, complementing mammalian cDNAs that restore mating (in a sir⁻ telomeric ura yeast strain) in addition to inducing a variegated phenotype are excellent candidates to represent mammalian homologues of SIR genes. We are currently applying this double selection screen to isolate the corresponding human and murine homologues of selected SIR genes.

SIR GENES AND POSITION EFFECT VARIEGATION

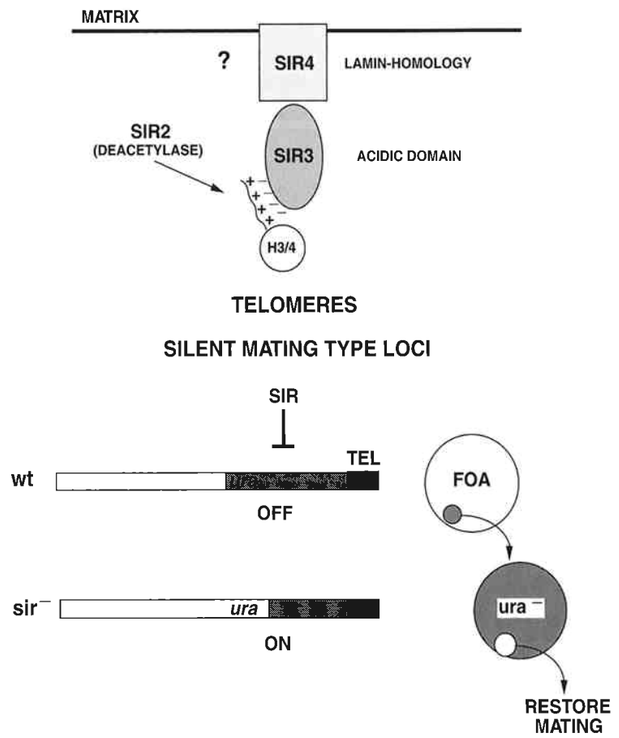

Fig. 3: SIR genes and position effect variegation

(c) Remodeling the chromatin structure *in vivo*

Angelika Lebersorger

The stable inactivation of clusters of genes through heterochromatinization or the activation of genes by decondensing higher order chromatin represent important control points to reprogram patterns of gene expression during early development. However, little is known about the mechanism(s) that underlie heterochromatinization. Recently, murine cDNA clones were isolated whose products are enriched in euchromatin (e.g. HMGI/Y) or heterochromatin (e.g. HP1). Furthermore, we have isolated a putative murine homologue of Su(var)3-9, a dominant suppressor of PEV in *Drosophila* that determines the spreading of heterochromatin (see above). We plan to offset the balance between euchromatin and heterochromatin by introducing null mutations for HMGI/Y and the putative Su(var)3-9 homologue in embryonal stem cells and mice. These studies will (i) advance our understanding of the mechanism(s) that form euchromatic and heterochromatic domains during early mouse development and (ii) allow the direct examination of the functional roles of these "general" coregulators of the chromatin structure in controlling gene activity.

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Synthetic Gene Transfer Systems

Group leader	Ernst WAGNER
Postdoc	Antoine KICHLER (since Sept. 94)
Postdoc	Wolfgang ZAUNER
PhD student	Ciaran MORRISON
Laboratory technician	Karl MECHTLER

Influence of lipid-membrane composition on disruption by amphipathic peptides

W. Zauner, K. Mechtler, C. Plank, A. Kichler and E. Wagner

Receptor-mediated endocytosis is an efficient means to deliver genes into mammalian cells. One of the limiting steps is the release of DNA from endosomes into the cytoplasm before it is degraded in lysosomes. Several classes of viruses have developed mechanisms to escape from endosomes by either fusing with the endosomal membrane (several enveloped viruses, e.g. influenza) or by destabilizing the endosomal membrane (e.g. adenovirus, poliovirus, rhinovirus). This membrane fusion/destabilization is triggered by the acidification of the internalized vesicles.

We wanted to mimick this pH-dependent action by synthetic peptides which were derived from two viruses. The peptides corresponded to the N-terminus of hemagglutinin subunit 2 of influenza virus (INF) and mutations thereof (see Plank *et al.*, 1994), and to the N-terminus of one of the capsid proteins (VP1) of human rhinovirus serotype 2 (HRV2). In addition, a rationally designed peptide was synthesized (GALA).

At acidic pH, all of these peptides have the potential to form an amphipathic alpha helix which is able to interact with membranes, while at neutral pH, alphahelix formation should be impeded by charge repulsion of the acidic amino acids present in the sequences. We have tested these peptides for their ability to disrupt artificial membranes of different composition [phosphatidylcholine (PC), phosphatidylserine (PS), PC/cholesterol (Chol), PC/phosphatidylethanolamine (PE)/PS/Chol, and PC/PE/Chol/sphingomyelin (Sphin)]and natural membranes (erythrocytes) in dependence of pH. With the exception of GALA, these peptides were able to release hemoglobin from erythrocytes. Surface charge seems to play a minor role for this effect, since neuraminidase-treated erythrocytes showed the same leakage behavior as untreated erythrocytes.

The extent of leakage of large unilamellar vesicles (LUV) induced by the peptides decreased in the order PC > PS > PC/Chol ~ PC/PE/PS/Chol ~ PC/PE/Chol/Sphin. For the different peptides, the ability to cause leakage from PC vesicles decreased from GALA>INF (and mutations)>HRV2. However, there were differences between these peptides regarding their ability to disrupt liposomes of different composition, e.g. GALA was unable to cause leakage of PC/Chol liposomes and was only weakly active with PC/PE/PS/Chol and PC/PE/Chol/Sphin. We have also tested these peptides for their ability to enhance gene transfer in several cell lines.

Development of a cancer vaccine for human malignant melanoma

M. Buschle, C. Denk, Z. Küpcü, M. Kursa, U. Scheer, S. Sharma, A. Sinski, and E. Wagner; Bender GmbH, Vienna
M. L. Birnstiel, M. Berger, E. Herbst, G. Maass, W. Schmidt, and T. Schweighoffer; IMP, Vienna. Kurt Zatloukal; University of Graz
G. Stingl, H. Korschan, F. Koszik, D. Pirkhammer, A. Schneeberger, and S. Schreiber; Dept. of Dermatology, University of Vienna Medical School

Vaccination with modified tumor cells is aiming at enhancing the immune response against tumor antigens expressed by the malignant cells. In murine models, different strategies for the development of cancer vaccines have been described. These include tumor cells transfected with genes coding for cytokines (such as IL-2, GM-CSF and others), co-stimulatory molecules (such as B7) or viral neo-antigens were able to induce tumor regression.

In malignant melanoma, several tumor associated antigens are known (e.g. the MAGE family of genes). Given the heterogeneity of tumor cells, it is likely that single tumor cell clones will not express the whole repertoire of tumor antigens. Therefore, vaccines constisting of bulk, unselected populations of melanoma cells may be more effective.

In a joint effort of the groups of Professor Birnstiel, Professor Stingl and our group, we were able to demonstrate that bulk IL-2 transfected, irradiated murine M3 melanoma cells can serve as a specific and efficient tumor vaccine both in a prophylactic and therapeutic model.

For the above-mentioned reasons, an autologous tumor vaccine genetically modified to secrete IL-2 appears to be a good concept for the treatment of patients with metastatic malignant melanoma. Essentially, preparation of such a vaccine consists of four steps: 1) a short term culture has to be established from melanoma metastases. 2) The culture is then transfected with a human IL-2 gene using transferrinfection followed by 3) irradiation with a dose preventing cell growth but not impairing cytokine production. 4) The vaccine is cryopreserved and shipped to the clinic.

A protocol for the isolation of melanoma cells from metastases was developed in collaboration with Professor Stingl´s group. We have been able to establish primary cultures from approximately 60% of resected malignant melanoma specimens. Using adenovirus-augmented transferrinfection, the melanoma cultures were transfected with an expression vector containing the human IL-2 cDNA under the control of the cytomegalovirus immediate early promoter/enhancer. In general, cytokine gene expression was highest between 48 and 72 hours following transfection, but was detectable more than 2 weeks following gene transfer. Cytokine production varied substantially among different isolates ranging from approximately 300 Units to greater 20,000 Units/10^6 cells/24 hours. We found that doses of up to 100 Gray (Gy) have only minor effects on cytokine gene expression. Although we did not observe cell growth following irradiation of cultures with >25 Gy, cultures used for the treatment of patients will be irradiated with a dose of 100 Gy as an additional safety margin. Finally, IL-2 expression was found to be relatively stable following cryopreservation. Taken together, a Phase I study using autologous, irradiated and unselected melanoma cells transfected by adenovirus-augmented transferrinfection with the human IL-2 gene appears feasible.

Development of a model for gene targeting in mammalian cells

C. Morrison, M. Cotten and E. Wagner

Using frameshift mutations of reporter genes (beta-galactosidase, luciferase) which result in defective expression from these genes, we sought to examine recombination-mediated repair as measured by recovered expression. Recovery of this expression was mediated by co-transfection of [non-expressing] repair fragments of the genes of interest, which contained the correct reporter gene sequence. The ability of cell lines (human A-549, murine NIH 3T3) to regenerate expression was first investigated in an episomal context, by co-transfection of the defective reporter together with the repair fragments. Various approaches were used to improve the levels of reporter gene expression: factors such as strandedness of the DNA (also inclusion of the *E. coli* RecA protein in transfection complex formation), the size of the repair fragment (homology length) and the effect of co-transfection of expression constructs for repair enzymes (XPC, XPG, Xrcc-1, Ku p70, Ku p86), which might be expected to increase the levels of the repair response, were examined in this regard. Different systems (cationic lipids, receptor-mediated transfection) were used for transfection of these cell lines.The defective reporter gene constructs were stably cloned into cell line hosts using G-418 selection. It has proved difficult to effect such repair in a chromosomal context, but these experiments present a model system by which requirements for non-selectable gene targeting may be analyzed.

The development of advanced receptor-mediated gene transfer vehicles has meant that gene delivery to EBV-transformed B lymphocytes has become possible; this system is being used to address the possibility of transfection-mediated repair of an existent genetic dysfunction, namely mutation in the *hprt* locus. Such a mutation presents a well-characterized and selectable target for repair. We hope to be able to use this empirical approach to repair (i.e., targeting) to gain information about what happens to DNA transfected into mammalian cells.

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Recessive Genetic Changes in Tumorigenesis: Positional Cloning Towards Tumour Preventing Genes

Group leader	Andreas WEITH
Postdoc (I.M.P.)	Paula STAPLETON (until May 94)
Postdoc (Boehringer Ingelheim Fonds)	Christoph LENGAUER (until July 94)
PhD student	Christoph BARNAS (until Aug. 94)
PhD student	Wilfried ELLMEIER (until June 94)
PhD student	Patrick ONYANGO
PhD student	Barbora LUBYOVA (since Oct. 94)
PhD student	Paola GARDELLIN (since Oct. 94)
Lab technician	Traudl HENN (until Sept. 94)
Lab technician	Karin PAIHA (since Oct. 94)

Introduction

The human chromosome 1pter-p35 region is suggested to encompass approximately 25-30 Mbp of DNA. As indicated by a strikingly high number of disease-specific chromosomal aberrations, several "disease genes" may be located in this genomic area. Particularly neoplastic diseases, such as primary hepatoma, phaeochromocytoma, Merkel cell carcinoma, mammary cancer, rhabdomyosarcoma, primitive neuroectodermal tumors (PNETs), neuroblastoma, or colorectal cancer display consistent rearrangements of the distal 1p region. In analogy to the distal part of the chromosome 11p arm, which has been designated MTACR1 (multiple tumor associated chromosomal region 1) owing to its frequent rearrangement in various cancers, we propose to designate the 1pter-p35 region MTACR2.

Strategies to approach disease-associated genes by genome analysis methods involve the pre-localization of candidate genes by either mapping disease-specific cytogenetic rearrangements or by genetic linkage in inherited forms of the respective disease. Both strategies require the availability of appreciably detailed physical or genetic maps.

While focusing on the analysis of distal chromosome 1p-associated genes that are involved in neuroblastoma, a genetic approach could not be taken since familial cases of this cancer type are very rare and mostly inaccessible to the scientific community. An approach to clone the gene(s) of interest, therefore, must involve a physical mapping approach. In this regard, numerous genomic mapping attempts in various laboratories have revealed that DNA of this particular region is poorly represented in genomic libraries, resulting in genetic and physical genome maps of insufficient resolution in this area. We had, therefore, set out to generate a most detailed 1pter-p35 specific physical map as a basis for positional cloning attempts.

Our experiments of the past several years to map the region of interest involved the generation of a large number of 1p36-specific DNA clones by microcloning (Martinsson *et al.*, 1989; Weith, 1994). Subsequent loss of heterozygosity analyses in neuroblastoma and hepatoma led us to determine a sub-region of 1p36 as the neuroblastoma consensus deletion (Weith *et al.*, 1989) and a hepatoma consensus deletion proximal to that (Simon *et al.*, 1990, Weith *et al.*, unpublished). However, in the absence of more precise mapping information neither the exact location and extent of these regions nor an increased number of specific markers could be established. We therefore initiated an approach that involved different physical mapping strategies to obtain more comprehensive information. Long-range restriction analysis using pulsed field gel electrophoresis as well as a "saturation cloning approach" of the entire 1p36 region with YAC and P1 clones was initiated. Upon isolation of YACs and P1s with 1p36 specific microclones, we established the use of these clones for molecular cytogenetic mapping by fluorescent in situ hybridization (FISH) (e.g. Lengauer *et al.*, 1994) in our laboratory. During the course of this year, we made extensive use of the long DNA clones to map them relative to the breakpoints of an extended panel of rearranged chromosomes 1, which were predominantly derived from tumor cell lines. We experienced that the combination of PFG fingerprint analyses and FISH to rearranged chromosomes provided an extremely powerful approach to very precisely localize both, DNA markers involving microclones,

cDNAs, Cosmids, P1 clones, and YACs as well as chromosomal rearrangements. We expect from this integral mapping approach a most detailed physical map which is a prerequisite to pinpoint and "positionally clone" the disease genes of interest.

A human chromosome 1p36 integral map

Figure 1 presents a schematic outline of the integral mapping approach. As a central resource, the pool of 1p36-specific microclones and a series of cDNA clones is used to perform loss-of-heterozygosity analyses, pulsed field gel electrophoresis (PFGE) fingerprinting, isolation of long DNA clones, and FISH analyses on metaphase chromosomes. The long clones will serve to map the respective loci relative to rearrangement breakpoints of marker chromosomes, thus creating a series of DNA "intervals" in the 1p36 region. The mapping to such intervals, which are bordered by physical rearrangement breaks, rather than the usual relative ordering of probes by double hybridization, proved to be very effective. Ultimately, cDNA probes of candidate genes may be isolated upon identification of appropriate regions within 1p36 and cloning either the corresponding CpG islands or via exon trapping or cDNA selection with long clones.

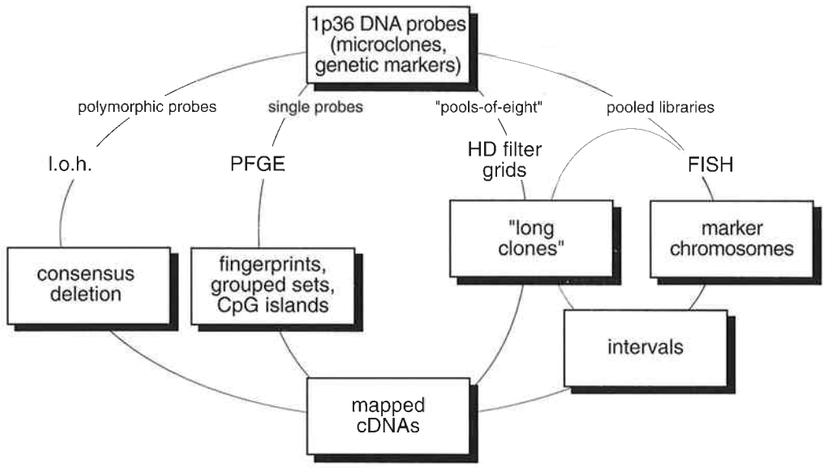

Fig. 1: Schematic outline of the integral mapping procedure. Four different physical mapping strategies (l.o.h.: loss-of-heterozygosity analyses, PFGE: pulsed field gel electrophoresis, HD filter grids: screening of membranes with robotically spotted colonies of YACs or P1 clones, FISH: fluorescent in situ hybridization) are combined to provide more detailed mapping data and a faster approach towards relevant genomic regions.

PFG fingerprinting: an extended panel of linked markers in grouped sets

Long range restriction analysis by hybridization of 1p36-specific DNA probes to standardized PFGE blots has established PFGE "fingerprint patterns" (see Weith *et al.*, 1993) for 102 single copy probes to date. Comparison of the individual fingerprints led to the identification of partially or completely identical patterns and consequently to "grouped sets" of probes, according to the GDB (genome database) nomenclature. The established sets of probes consist of two to eleven markers. To date, 17 grouped sets are determined containing altogether 72 markers; 14 grouped sets map to the 1pter-p36.12 region, the remaining three are localized in the interval 1p36.11-p35. Since most of the grouped sets contain one or more genetically mapped markers, the relative order of these groups could be established according to the genetic mapping information (see bracketed areas "GS1" to "GS17" of the integral map in **fig. 2**). Furthermore, upon linking YAC and P1 clones to markers of the individual grouped sets, the respective FISH analysis provided additional mapping information on the relative order (see below).

When we set out to generate a restriction map across the 30 Mbp area of interest, we designated a total of 102 markers sufficient for such an attempt. Provided an even distribution, the markers would be spaced at approximately 300 kbp intervals. However, upon completion of the PFGE fingerprint analysis it became evident that the available number of probes merely facilitated the establishment of several extended grouped sets with gaps in between instead of a continuous map. Several reasons may be envisaged that accounted for the discontinuity. First, the genomic region targeted may indeed be larger than originally anticipated. Second, the DNA probes may not be distributed as evenly as suggested. Finally, rare cutter restriction digests of the region analyzed may generate restriction fragments which are too small to be used for linkage analysis.

Whilst our calculation of the size of the genomic region coincides well with other estimates, the two latter suggestions indeed may apply. We observed that some grouped sets contained up to four times as many markers as other, similarly sized ones. Moreover, we found a clustering of six VNTR and RFLP markers to a single 300 kbp *Mlu I* fragment, which clearly demonstrates a non-statistical distribution of markers.

With respect to the sizes of restriction fragments, we observed that indeed a distinct subset of probes generated a series of very small, strikingly equally sized hybridization bands. We have reported previously that these patterns indicate the presence of adjacent, closely apposed CpG islands (Ellmeier *et al.*, 1992; Ellmeier *et al.*, in preparation). It is conceivable that such closely adjacent clusters of restriction sites, as they are found in CpG islands, make it increasingly difficult to physically link loci across such a region. It is known also from another investigation that chromosome 1p36 apparently is enriched in CpG islands or island-like sequences (Craig and Bickmore, 1994); we may therefore suggest that the gaps in between the grouped sets correspond to regions rich in CpG islands.

As another important consequence of the PFGE mapping analysis presented here, we may relocate and redefine a "neuroblastoma consensus deletion", which we previously localized to 1p36.2-p36.1 (Weith *et al.*, 1989). Those polymorphic DNA markers that consistently displayed allelic deletions wherever loss of heterozygosity was detectable (D1S94 - D1S97), could be mapped to the two grouped sets GS2 and GS3 (**Fig. 2**). Both map to the distal 1p36.3 band; hence we may suggest that a consensus deletion in neuroblastomas may be located more distally than originally assumed. This is supported by an independent in situ hybridization analysis of a series of distal 1p probes (van Roy *et al.*, 1993), mapping these probes very distal to 1p36.3.

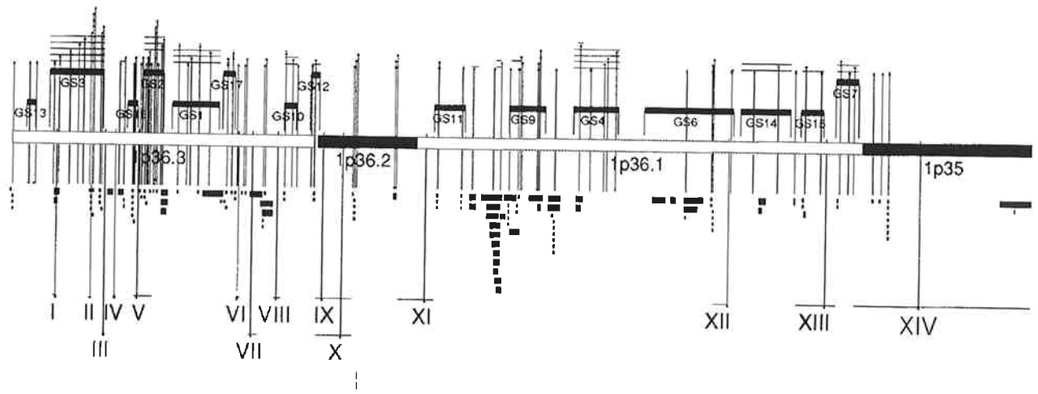

Fig. 2: An Integral Map of MTACR2 (the distal 30 Mbp of the human chromosome 1p arm). The chromosome region 1pter-p35 is represented by the grey and black horizontal bar with indications of the chromosomal sub-bands. Above, the single dots with vertical reference lines represent microclone or cDNA probes. Small horizontal lines indicate the possible locations of the respective markers. Bracketed regions with designations GS1-GS17 show the areas covered by grouped sets of probes. Below the chromosome bar, two types of long clones are displayed at their appropriate position: YAC clones (thick horizontal bars) and P1 clones (small horizontal bars). The cloned fragments are drawn to scale in order to indicate the coverage of the region. Roman numbers with vertical reference bars refer to the chromosomal breakpoints listed in table 1.

"Binning" of long DNA clones: FISH of YAC and P1 clones to rearranged chromosomes

(in collaboration with R. Elaswarapu, HGMP Resource Centre, Cambridge, and F. Francis and H. Lehrach, ICRF London)

Using pools of radiolabeled microclones and other 1p36-specific probes, we have isolated 348 YACs and 241 P1 phage clones to date. Using FISH mapping to metaphase chromosomes of normal karyotypes we could so far identify 67 YACs and 138 P1s to be specific for 1p36. As reported earlier, approximately 40% of the YAC clones are chimeric; however, only two P1 clones were found to generate fluorescent signals both on

chromosome 1 and another chromosome. Using PFGE, the mean size of the 1p36 YACs was determined to be 300 kbp, ranging from 80 kbp to 900 kbp; the P1 clones had an average length of 75 kbp, ranging from 22 kbp to 93 kbp.

When we set out to establish a relative order of the long DNA clones, we did not rely on double hybridization as a technique, since we apprehended a number of wrong assignments owing to the large number of clones and small size of the region targeted. We rather mapped the probes by FISH relative to cytogenetically aberrant chromosomes with breakpoints within the 1pter-p35 region, thus creating a series of intervals or "bins", each bordered by two distinct and invariable rearrangement breakpoints. **Table 1** lists the aberrant chromosomes used for this purpose. This interval mapping allowed an unequivocal ordering of all markers into 15 distinct intervals within the 1pter-p35 region (**Fig. 2**). Altogether 53 YACs, 97 P1 clones, and six Cosmids were included in this map to date.

As the YAC, P1 and Cosmid clones were homologous to individual microclones or cDNA clones, these markers could concomitantly be included into the map. In cases where these markers had been included into grouped sets of probes (see above), long clones could also be associated with these groups, thus providing a link between the cloned DNA and the long range restriction pattern. This is eventually expected to yield extensive information on any disease-related DNA sequence rearrangements in the respective regions.

Most importantly, the selection of aberrant chromosomes as "binning tools" is expected to directly involve the approach towards important chromosomal regions: as listed in **table 1**, the majority of rearrangements are derived from neuroblastoma or other tumor karyotypes. FISH analysis relative to such rearrangements may consequently involve the direct identification of long clones that map to the neuroblastoma consensus deletion or even of clones that may span one of the balanced translocation breakpoints. As shown below, we succeeded in both, redefining the neuroblastoma consensus deletion and assigning a number of additional markers to it, as well as in the identification of a YAC clone that spans the breakpoint of a balanced translocation in a primitive neuroectodermal tumor.

Redefining the neuroblastoma consensus deletion: Evidence for a very distal susceptibility locus

in collaboration with P. White, J. Biegel, and G. Brodeur, Children's Hospital, Philadelphia; C. Hussussian and N. Dracopoli, NCHGR, Bethesda

Both, PFGE fingerprint analysis and FISH mapping, now suggest that those RFLP markers, which consistently detected allelic deletions in many neuroblastoma tumors, indeed are located in 1p36.3, i.e. far more distal than previously suggested. Two more lines of evidence point towards a very distal location of the neuroblastoma consensus deletion: We discovered a very small interstitial deletion in a neuroblastoma-derived cell line (between breakpoints I and VIII in **Tab. 1** and **Fig. 2**), whose proximal border is still located in 1p36.3. Furthermore, the constitutional genotype of a neuroblastoma patient also displays a small interstitial deletion that partially overlaps the deletion of the neuroblastoma-derived cell line (see breakpoints III and X in **Tab. 1** and **Fig. 2**). We designate the smallest region of overlap of these two deletions the neuroblastoma consensus deletion (III-VIII in **Fig. 2**), whose location may now be 1p36.32-p36.31. The size of this critical region may be even further reduced upon the assignment of the distal deletion breakpoint IV in a neuroblastoma cell line (**Tab. 1**, **Fig. 2**) as the distal border of the consensus deletion.

The significance of balanced translocations: breakpoints in a neuroectodermal tumor cell line and a neuroblastoma cell line map to the consensus deletion, embryonal rhabdomyosarcomas don't

in collaboration with P. Ambros, CCRI Vienna, D. Shapiro, St. Jude's Hospital, Memphis, D. Sheer, ICRF London, and P.White and G. Brodeur, Children's Hospital, Philadelphia

Whilst mapping the long DNA clones to aberrant chromosomes, we made another most important observation: two balanced translocation breakpoints in neuroectodermal tumors (breakpoints VI and VII in **Tab. 1** and **Fig. 2**) were localized to the neuroblastoma consensus deletion, thus confirming its newly assigned position; the t(1;13) breakpoint of an embryonal rhabdomyosarcoma (breakpoint XIV in **Tab. 1** and **Fig. 2**), however, was located more proximal in 1p35. Additional evidence for more proximal rearrangements in embryonal rhabdomyosarcomas was gained from the analysis of another tumor cell line of this cancer type (not shown). This may indicate the specificity of a putative tumor susceptibility gene in 1p36.32-p36.31 for neuroectodermal tumors.

The FISH analysis on chromosomes of the PNET cell line (breakpoint VII in **Tab. 1** and **Fig. 2**) unequivocally placed a 290 kbp YAC clone directly across the

breakpoint (**Fig. 3**). Fluorescent signals were obtained on both derivative chromosomes in this case, indicating that the DNA contained in the YAC was discontinuous in the rearranged chromosomes. This was not seen for two cloned YAC probes immediately centromeric of this YAC, though these probes had been detected with the same microclone (see **Fig. 3**). Also, a P1 clone immediately telomeric did not display a "split" pattern of hybridization. It was therefore evident that the rearrangement is confined to the DNA sequence contained in the 290 kbp YAC clone. Further work is in progress to sub-clone the YAC into Cosmids and to isolate corresponding cDNAs via cDNA amplification/selection.

With respect to the balanced translocation in the neuroblastoma cell line studied (breakpoint VI in **Tab. 1** and **Figs. 2, 3**), we assigned its breakpoint telomeric to the one in the PNET cell line; however, merely two P1 clones map to the interval between the two breakpoints (**Fig. 3**). Hence, despite a minimal distance between the two of approximately 150 kbp, still the same gene may be affected by the rearrangements. The molecular cloning of the neuroblastoma translocation breakpoint is currently in progress.

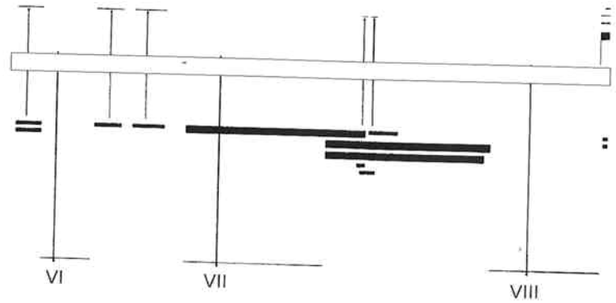

Fig. 3: A sub-region of the neuroblastoma consensus deletion containing the loci for the two balanced translocation breakpoints VI and VII. The grey horizontal bar refers to the chromosomal DNA; dots above the bar with vertical reference lines represent four microclone probes and a cDNA probe; thick horizontal bars below indicate the position of YAC clones; smaller horizontal bars describe position and extent of P1 clones. All cloned fragments are drawn to scale (The clone spanning the breakpoint VII represents 290 kbp of DNA). The space between VI and VII is drawn at an arbitrary size since the exact distance is not known to date.

No.	type	partial karyotype	breakpoint analyzed
I	NB	del(1)(p36.3)	distal deletion breakpoint
II	n.a.	t(1;22)(p36;p11)	translocation breakpoint
III	NB[a]	del(1)(p36.3)	distal deletion breakpoint
IV	NB	del(1)(p36.3-p11)	distal deletion breakpoint
V	MR	t(1;4)(p36.1;p14)	translocation breakpoint
VI	NB	t(1;15)(p36.3;?)	translocation breakpoint*
VII	PNET	t(1;X)(p36.3;p12)	translocation breakpoint*
VIII	NB	see no. I*	proximal deletion breakpoint
IX	NB	see no. VI	duplication breakpoint
X	NB[a]	see no. III	proximal deletion breakpoint
XI	MCA	t(1;4)(p36.11;p15.32)	translocation breakpoint
XII	n.a.	t(1;8)(p36.11;q22)	translocation breakpoint
XIII	NB	del(1)(pter-p36.11)	proximal deletion breakpoint
XIV	eRH	t(1;13)(p35;q23)	translocation breakpoint*

Tab. 1: Chromosomal breakpoints for FISH mapping. Tumor cell lines, lymphoblastoid cell lines, or fibroblast cell lines were used to prepare metaphase spreads for FISH mapping. The partial karyotypes indicated refer to the relevant changes of chromosome 1 in the respective cell line.
Roman numbers refer to the designations in the map of figure 2. **NB:** neuroblastoma, **MR:** mental retardation, **PNET:** primitive neuroectodermal tumor, **MCA:** multiple congenital abnormalities, **eRH:** embryonal rhabdomyosarcoma, **n.a.:** not ascertained. **a:** constitutional case, *: balanced translocation

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Nuclear Oncoproteins and Transcription Factors in Hematopoietic Cell Differentiation

Group leader	Martin ZENKE
Postdoc	Petr BARTUNEK
Postdoc	Guido BOEHMELT (until June 94)
PhD student	Karoline BRIEGEL
PhD student	Sandra DIEBOLD (since Sept. 94)
PhD student	Jaime MADRUGA
Visiting scientist	Nicolas KORITSCHONER (since Nov. 94)
Technician	Gabi STENGL

Introduction

Differentiation 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 group studies the impact of various transcriptional regulators on the differentiation potential of such progenitors. Retroviral oncogenes are employed to transform early hematopoietic progenitor cells of chicken which can then be amplified and propagated in vitro under well defined culture conditions. In the avian system, such oncogene transformed, non-established cell strains retain their capacity to undergo apparently normal terminal differentiation. This approach is used to study molecular mechanisms of red blood cell differentiation in vitro. Additionally, it allowed us to develop a powerful in vitro differentiation system for antigen-presenting dendritic cells.

1. Transcriptional regulators of red blood cell differentiation

1.1.erbA, RXR and RAR

Petr Bartunek

In 1994 we continued our studies of the activity of the c-erbA/T3 receptor (T3R) and the erbA-related receptors RAR and RXR (for all-*trans* retinoic acid [RA] and 9-*cis* RA, respectively) in red blood cell differentiation. Both primary ts-oncogene transformed and normal TGFα- or SCF-dependent red blood cell progenitors (see Report of Hartmut Beug) were used.

It was found that thyroid hormone (T3), RA and 9-c*is* RA accelerate erythroid cell differentiation, with T3 (if applied individually) being the most effective. However, maximal differentiation induction was achieved when T3, RA and 9-c*is* RA were applied simultaneously. Additionally, the use of a series of synthetic RXR-specific ligands (in collaboration with A. Levin, Hoffmann-La Roche, Nutley, USA and P. Chambon, Faculté de Medecine, Strasbourg, France) now allowed us to dissect the individual contribution of the RXR receptors on red blood cell differentiation. Our results are further extended by ectopic expression of dominant negative RXR (dnRXR) versions (in collaboration with H. Stunnenberg, EMBL, Heidelberg, Germany and P. Chambon, Faculté de Medecine, Strasbourg, France) which delayed differentiation. Thus, our studies uncover an important function of the RXR receptor in red blood cell development.

1.2 erbA target genes

Karoline Briegel, Nicolas Koritschoner and Gabi Stengl

Previous work demonstrated the impact of the erbA proteins on erythroid cell differentiation. So far, three red cell-specific erbA target genes [carbonic anhydrase II (CAII), the anion transporter band3 and aminolevulinic acid synthase (ALA-S); Zenke *et al.*, 1988, 1990] are known. However, their expression pattern can explain only part of the erbA-specific phenotype (Fuerstenberg *et al.*, 1992). Therefore we spec-

ulate that other targets must exist which should play a more decisive role in red blood cell maturation. By using conventional differential cDNA cloning and PCR-based differential display, several erbA-regulated genes were identified. One of them encodes a Krüppel-related Zn finger protein and therefore a putative transcriptional regulator. Other erbA target genes are potentially involved in protein biosynthesis and/or protein folding. Additionally, while some of the genes are regulated by both the T3R and the v-erbA oncoprotein, others are not. Thus, the T3R and the v-erbA oncoprotein share common target genes, but also act on distinct sets of genes. The impact of these potential erbA target genes on self-renewal and differentiation of red blood cell progenitors is currently being studied.

1.3 GATA transcription factors

Karoline Briegel and Petr Bartunek, in collaboration with D. Engel, Northwestern University, Evanston/ Chicago, USA

The GATA factors are a family of Zn finger transcriptional regulators which display a highly conserved tissue-specific and temporal expression pattern in all eukaryotes. Interestingly, three members of this family, GATA-1, GATA-2 and GATA-3, are coexpressed at different and changing ratios during normal red blood cell differentiation. Our previous work established that the precise quantitative balance of the GATA proteins plays an important role in influencing the developmental decisions available to erythroid progenitor cells, i.e. to undergo either self-renewal or terminal differentiation. Accordingly, ectopic expression of GATA-2 (or of a hormone-activated GATA-2/ER fusion protein) in red cell progenitors induced their self-renewal capacity and inhibited differentiation (Briegel *et al.*, 1993). Thus, the developmentally controlled down-regulation of endogenous GATA-2 appears to be a prerequisite for their normal terminal differentiation. By using a similar approach we have now studied the activity of GATA-1 factor in red blood cell differentiation.

The available expression data of GATA-1 in immature, self-renewing erythroid progenitors initially presented an enigma. In these cells, GATA-1 mRNA is abundantly expressed, while at the same time the expression of the β-globin gene, a presumptive direct target of GATA-1 activity, is relatively low. Moreover, gene targeting and rescue experiments demonstrated a role for GATA-1 in differentiation of erythroid progenitors rather than in their self-renewal capacity. These apparently disparate observations prompted us to investigate whether activity of the GATA-1 factor itself might be regulated during terminal erythroid differentiation.

It was found that self-renewing erythroid progenitor cells express high levels of both GATA-1 mRNA and protein. However, at this immature stage the majority of GATA-1 factor is confined to the cytoplasm. Most importantly, upon differentiation induction essentially all GATA-1 protein accumulates in the nucleus and avidly binds to DNA. In attempting to mimic this developmentally controlled translocation mechanism, we ectopically expressed a conditional GATA-1/ER fusion protein (whose nuclear translocation is controlled by hormone) in red blood progenitor cells. It was found that hormone-activated GATA-1/ER protein translocates into the nucleus and accelerates red cell differentiation. We propose a model in which regulation of the intracellular distribution of a pre-existing lineage-specific transcription factor represents a paradigm for the rapid induction of a differentiation program.

1.4 The coordinate action of erbA, GATA-1 and c-myb during red blood cell differentiation.

Petr Bartunek and Karoline Briegel, in collaboration with M. Dvorak, Institute of Molecular Genetics, Prague, CR

While activation of the T3R or of the conditional GATA-1/ER fusion protein induces expression of a variety of erythroid-specific genes, transcription of the c-myb proto-oncogene gets downregulated. Additionally, as demonstrated by many investigators, high and constitutive c-myb expression efficiently inhibited red blood cell maturation. Thus, a coordinate action of erbA, GATA-1 and c-myb might be a prerequisite for normal erythroid cell differentiation. We now provide support for this hypothesis.

It was found that GATA-1 protein directly interacts with regulatory sequences of the c-myb gene. By employing various strategies, 3 major GATA-1 binding sites were identified within the c-myb promoter. GATA-1 protein effectively binds to these sites *in vitro* as demonstrated by electrophoretic mobility shift assay. One of the GATA-1 binding sites resides in a promoter proximal position within a sequence element which is extremely conserved during evolution. Furthermore, the hormone-activated T3R modulates GATA-1 binding to one of the more distal GATA-1 binding sites, suggesting that this site might be important for c-myb downregulation mediated by T3R.

Our experiments therefore uncover multiple erbA, GATA-1 and c-myb interactions within the gene expression network which governs red blood cell differentiation.

2. v-relER transformed chicken bone marrow cells differentiate into fully competent antigen-presenting dendritic cells.

Guido Boehmelt, Jaime Madruga and Nicolas Koritschoner, in collaboration with P. Enrietto, SUNY, Stony Brook/New York, USA

In 1994 we continued our studies on the differentiation potential of chicken bone marrow cells transformed by a conditional v-rel estrogen receptor (ER) fusion protein v-relER (Boehmelt *et al.*, 1992).

v-rel, the oncogenic version of c-rel transduced by the avian retrovirus complex REV-T/REV-A, belongs to the NF-kB/rel/dorsal transcription factor family. Initial evidence suggested that v-rel contained within the REV-T/REV-A virus induced a disease of lymphomatous origin, while other studies indicated that the disease was a reticuloendotheliosis, affecting cells associated with endothelia of blood vessels and with sinusoids of spleen, kidney, liver and lymphoid organs. Additionally, the in vitro target cell for v-rel-specific transformation was classified as lymphoid, but was found to express also surface antigens specific for myeloid cells. Thus, the identity of the v-rel target cell remained obscure. The availability of the conditional v-relER now allowed us to examine the differentiation potential of v-rel-transformed cells by releasing the v-rel-induced differentiation arrest and following their fate of differentiation.

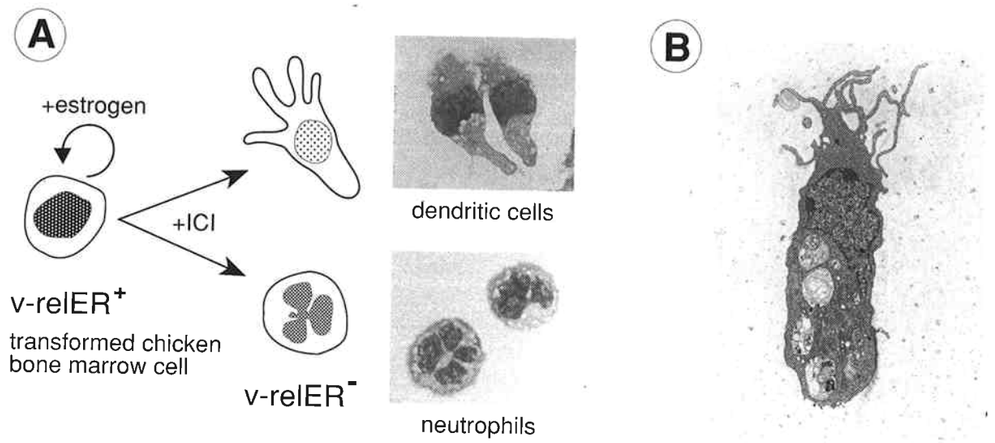

Fig.1: Differentiation potential of v-relER transformed chicken bone marrow cells.
(A) v-relER progenitor cells are grown in the presence of estrogen and induced to differentiate in vitro under appropriate culture conditions by administration of the estrogen antagonist ICI 164.384 (ICI). Differentiation into dendritic cells and cells resembling polymorphonuclear neutrophils is shown.
(B) Electronmicrograph of dendritic v-relER cell (in collaboration with H. Schwarz, Max-Planck-Institute for Developmental Biology, Tübingen, Germany).

This work led to the unexpected discovery that clonal populations of v-relER cells have the potential to differentiate into fully competent antigen-presenting dendritic cells and into cells reminiscent of polymorphonuclear neutrophils (**Fig. 1**).

Dendritic cells are found at various locations within the organism and represent a cell population whose function it is to capture antigens, migrate to lymphoid organs and present the processed antigens to lymphoid cells. Accordingly, several morphological and functional properties of differentiated v-relER cells support our conclusion. Dendritic v-relER cells exhibit an elongated, bipolar morphology, form lamellipodia or veils, and are highly motile in culture (**Figs. 1** and **2**). They exhibit a low to moderate phagocytic activity, express high levels of MHC class II and stimulate T cell proliferation in a primary mixed lymphocyte reaction (Boehmelt *et al.*, 1995a).

Differentiation of v-relER cells into cells resembling neutrophils is of particular interest, since neutrophil differentiation in vitro under well defined culture conditions has so far not been achieved. Yet, to date characterization of the neutrophil v-relER cells is mainly based on morphological criteria and a more thorough characterization of this cell type still has to be performed. We also note that undifferentiated v-relER progenitor cells exhibit a number of B-lymphoid determinants in line with previous studies on v-rel transformed cells; they contain rearranged immunoglobuline genes, and express surface IgM and the B cell-specific activator protein BSAP (in collaboration with P. Dörfler, IMP, Vienna). Thus, our experiments support

the notion that v-relER, and presumably also v-rel, transform an early hematopoietic precursor for dendritic cells and neutrophils, which also expresses B-lymphoid markers.

Our current efforts are directed towards studying the gene expressional repertoire of dendritic v-relER cells by PCR-based differential display. This approach should lead to the identification and cloning of dendritic cell-specific genes and gene products.

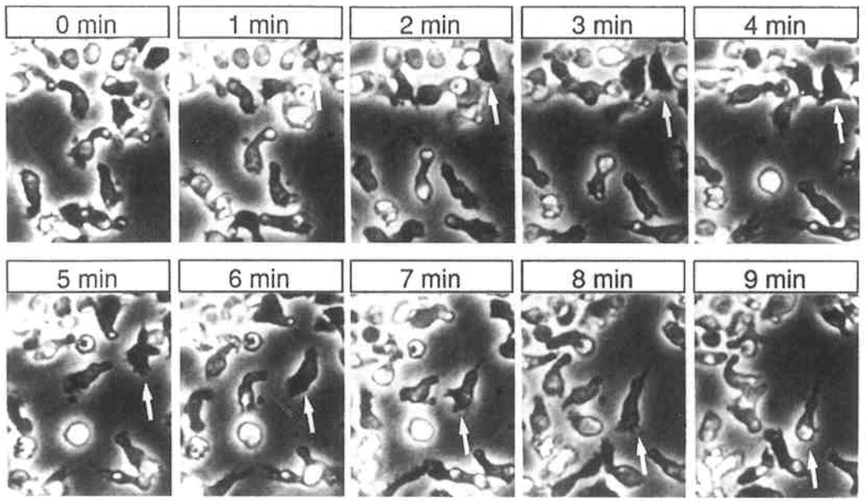

Fig.2: Time-lapse cinemicroscopy of dendritic v-relER cells.
Dendritic v-relER cells are highly motile in culture as revealed by time-lapse cinemicroscopy. Cells migrate about 4-5 times their body length within 10 minutes (arrow points to one representative cell).

3. Gene transfer into antigen-presenting dendritic cells.

Sandra Diebold

Recently we also developed techniques for efficient gene transfer by receptor-mediated endocytosis into dendritic cells of chicken, mouse and human (in collaboration with M. Cotten and M. L. Birnstiel, IMP, Vienna; E. Wagner, Bender & Co, Vienna; B. Dörken and A. Pezutto, MCD, Berlin, Germany). These studies will ultimately enable us to introduce and express in dendritic cells e.g. tumor cell-specific epitopes and therefore might provide a basis for immunotumor therapy by gene therapy.

Publications during the year

Beug, H., Bartunek, P., Steinlein, P. and Hayman, M. (1994). Avian hematopoietic cell culture. In vitro model systems to study oncogenic transformation of hematopoietic cells. *Methods in Enzymology*, in press.

Boehmelt, G., Madruga, J., Dörfler, P., Briegel, K., Schwarz, H., Enrietto, P. and Zenke, M. (1995a). 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. (1995b). v-rel protects transformed chicken bone marrow cells from apoptosis: A study with the conditional v-rel estrogen receptor fusion oncoprotein v-relER. *Proc. Avian Immun. Res. Group* **1994**., in press.

Boehmelt, G., Ulrich, E., Kurzbauer, R., Mellitzer, G., Bird, A. and Zenke, M. (1994). Structure and expression of the chicken retinoblastoma gene. *Cell Growth Diff.* **5**, 221-230.

Briegel, K., Bartunek, P., Stengl, G., Lim, K.-C., Beug, H., Engel, J.D. and Zenke, M. (1995). Nuclear translocation of GATA-1 is an important event in triggering red blood cell differentiation. Submitted.

Pollerberg, G. E., Kuschel, C. and Zenke, M. (1994). Generation of cell lines from embryonic quail retina capable of mature neuronal differentiation. *J. Neurosci. Res.*, in press.

Other references

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

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

Fuerstenberg *et al.* (1992). *EMBO J.* **11**, 3355-3365.

Zenke *et al.* (1988). *Cell* **52**, 107-119.

Zenke *et al.* (1990). *Cell* **61**, 1035-1049.

Animal House

Scientific Coordinator	Erwin F. WAGNER
Veterinary Doctor	Karl SCHELLANDER (Univ. Vienna)
Chief Technician	Norma HOWELLS (until Dec. 94)
Chief Technician	Melanie KING (since Nov. 94)
Technician	Mijo DEZIC
Technician	Ryzard KUPINSKI
Technician	Christian THEUSSL
Technician	Erika WEISSENGRUBER

The animal house provides technical support to the scientists and at present holds various in-, out- and hybrid strains of mice, out-bred chickens, random-bred rabbits and toads.

Current production of animals bred in-house is as follows: approximately 360 stock mice per week, 70 chicken embryos per week and 10 chickens per week. Additional mice for short-term studies, toads and rabbits are purchased from external suppliers due to economic reasons.

The animals listed above are used in the following studies: various mouse projects (see reports of Erwin Wagner, Denise Barlow, Thomas Jenuwein, Gerhard Christofori, Meinrad Busslinger, Andreas Weith and Max Birnstiel), and in chicken leukaemia studies (see reports of Hartmut Beug and Martin Zenke). The rabbits are used by various Groups and the toads by the Max Birnstiel Group.

A new mouse room, together with a new microinjection laboratory were built and are now in operation due to an increase in experimental mouse studies. In addition to these, a new rabbit facility is under construction, which will be operational in early 1995.

The technical procedures offered by the animal house are performed to a high standard, thus ensuring that any distress caused to the animal is kept to a minimum. Procedures include the collection of mouse, rabbit and chicken blood and the collection of embryonic and adult tissues for DNA and RNA analysis. Various substances are administered to the animals which include hormones and anaesthetic agents. Surgical procedures such as tumour implantation, vasectomy and hysterectomy are routinely performed together with the subsequent resuscitation of potential transgenic or chimaeric mice.

Protein Modeling

Scientist	Anton BEYER
PhD-Student	Aron MARCHLER-BAUER (on temporary leave since 1.6. 94)
Diploma Student	Wolfgang BITOMSKY (since 1.3. 94)

Introduction

Our main interest 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. In addition to 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.

Bianca Habermann uses statistical methods to find correlations between the temperature factor from crystal structures, surface accessibility of a given residue, and an empirical energy per residue, caculated using Aron Bauer's potential.

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.

Wolfgang Bitomsky performs molecular dynamics simulation studies to investigate structural differences and the flexibility of ras.p21.GDP and ras.p21.GTP.

Sequence-to-structure matching of globular proteins

Aron Marchler-Bauer

The alignment of protein sequences has become a valuable and widely-used tool in molecular biology. Recent advances have made possible the alignment of protein sequences to protein structures and vice versa, sometimes termed the inverse protein folding problem, or, simply, threading.

To assess the usefulness of this approach we have developed and compared threading methods which either (a) are a variation of the dynamic programming scheme and use sequence information of the target structure, too, or (b) use a more general scheme to avoid using sequence information from both proteins.

a) Simple threading algorithms that use the dynamic programming approach are easy to implement and are sufficiently fast to be used for database screening. Their drawbacks are (I) low sensitivity for spurious matches that cannot be detected on the sequence-to-sequence comparison level alone, and (II) the need for gap insertion and gap elongation penalties. There is no justification for the actual size of gap penalties other than the quality of the resulting alignments. The major problem is sensitivity, since an excellent detection ability is expected from threading approaches. It is evident that this feature can be improved only by the quality of the assessment criterion, which is a potential of mean force, derived from known protein structures.

b) Approaches different from dynamic programming schemes are designed to use sequence information from one protein and structure information from the second molecule solely. Algorithms were devised which either (I) calculate every single element of the alignment matrix, or (II) use short blocks of sequence that are assigned to blocks of structure, making use of a large number of block-to-block interactions. While all these approaches are, in principle, highly sensitive, they are seriously hampered in performance and are not guaranteed to calculate the correct (optimal) alignment in reasonable time. The problem of finding the optimal threading has turned out to be NP-complete, as is structure prediction in general (Lathrop, 1994). Nevertheless algorithms may be found which calculate approximated solutions of high quality rather fast.

All the approaches described above will depend on the quality of the underlying potentials of mean force, used for sequence-on-structure matches. We have continued work to improve these potentials which now consist of pairwise interaction terms, of solvation potentials and terms accounting for dihedral backbone angles. The set of structural data used to derive potential parameters was increased significantly by allowing for even close homologs and accounting for the redun-

dancy in a proper fashion. This has improved the statistics and smoothed the potential functions. Their quality is assessed by a simple non-gapped threading experiment, where all proper globular domains are recognized with high reliability and the average Z-score of the native match is used to evaluate the performance of the potentials.

Molecular dynamics simulation studies of ras.p21.GTP and ras.p21.GDP

A. Beyer, W. Bitomsky

Ras.p21 belongs to the class of small nucleotide-binding proteins. It binds either GTP or GDP and one metal ion which is essential for proper function. Members of this family have a high sequence and structural homology at the GTP binding site. Ras p21 is involved in several signal transduction pathways. There exist two possible states of the protein: the active GTP-bound form (on-state) and the inactive GDP-bound form (off-state). Point mutations in ras.p21 often cause an accumulation of the GTP-bound form which in turn leads to severe distortions in the regulation of cell growth and is responsible for a high fraction of human cancers. Knowing the three dimensional structure from x-ray experiments, some of these effects can be explained in a rational way (Wittinghofer *et al.*, 1993).

The structure is made up of a six stranded sheet, five alpha helices and ten loops. Two of these loops are important for GTP hydrolysis. A large conformational change takes place in two regions of the molecule (switch I , residues 30-38 and switch II, residues 61-77) during hydrolysis. Molecular dynamics calculations on GTP-and GDP-bound protein including solvation were performed to get some insight into the structural flexibility of these regions and into the dynamic behavior of the whole molecule. We are currently analysing the molecular dynamics trajectories of these two simulations.

Publications during the year

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

Other references

Lathrop, R. H. (1994). The protein threading problem with sequence amino acid interaction preferences is NP-complete. *Protein Engineering* **7**, 1059-1068.

Wittinghofer, A., Franken, S. M., Scheidig, A. J., Rensland, H., Lautwein, A., Pai, E. F. and Goody, R. S. (1993). Three-dimensional structure and properties of wild-type and mutant H-ras-encoded p21. In: The GTPase superfamily, *CIBA Foundation Symposium* **176**, Wiley 1993.

Service Department

Scientist	Gotthold SCHAFFNER
Technician	Elisabeth AIGNER
Technician	Ivan BOTTO
Technician	Robert KURZBAUER
Technician media kitchen	Gabriele BOTTO
Technician media kitchen	Christa CZAPKA

Introduction

The Service Department offers a variety of rapid services to scientists within the IMP as well as to a few scientists outside the Institute. The most time-consuming services are DNA sequencing and oligonucleotide synthesis.

We also give help in designing immunization protocols and test titers of antisera. This is only possible with the help of the Animal House staff and their animal breeding and keeping facilities.

The reliability and quality of cell culture media and other solutions prepared by our Media Kitchen staff is vital for the continuity of cell culture and other high standard work done in the Institute.

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

Oligonucleotide Synthesis

In 1994, we increased again the number of oligonucleotides synthesized to about 1900 (**Fig. 1**). With the help of the new 4 column synthesizer (PERKIN-ELMER/APPLIED BIOSYSTEMS 394), we now deliver oligonucleotides within 24 hours on normal working days. It is now possible to synthesize up to 7 oligonucleotides simultaneously.

This year we tested oligonucleotides fluorescently labeled with 6-FAM (P-E/ Applied Biosystems) as an alternative to radiolabeled primers to detect heterozygotic genes. Together with Oskar Smrzka from Denise Barlow´s laboratory, we obtained convincing results that indicate the usefulness of these oligonucleotides (**Fig. 2**). The analysis was performed on a P-E/ Applied Biosystems 393 A Sequencer with GENE SCAN software.

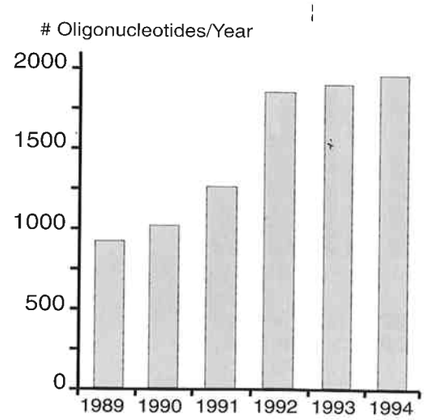

Fig. 1: Total amount of oligonucleotides synthesized per year.

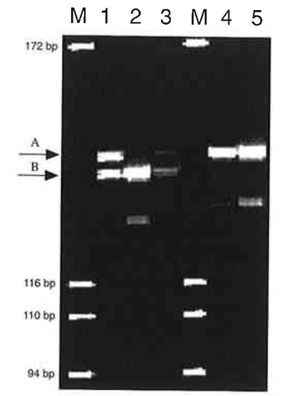

Fig. 2: PCR based screen with fluoresceine (6-FAM) labeled oligonucleotides for 2 alleles of IGF2R locus in human DNA samples prepared from peripheral blood leucocytes. There is a difference of 4 nucleotides in PCR fragment length between allele A und B.
M : Size marker labelled with rhodamine. Lane 1 to 5 represent individual samples. Lane 1 shows both alleles and 2 to 5 either allele A or B.

Sequencing

IMP scientists have come to rely less on conventional sequencing using SEQUENASE (USB) and the sequenase protocol with radioactive dATP. The numbers in **Fig. 3** clearly show this trend: 3600 + samples sequenced with fluorescently labeled dideoxynucleotides vs. 1300 + samples sequenced with radiolabelled dATP. We have shown that the results obtained with the automated 393A sequencer (P-E/Applied Biosystems) and the DyeDeoxy Taq sequencing protocol are very reliable. The availability of the data on computer and the ease of aligning sequences makes automated sequencing very attractive. We expect a further shift towards sequencing with nonradioactive labeled nucleotides.

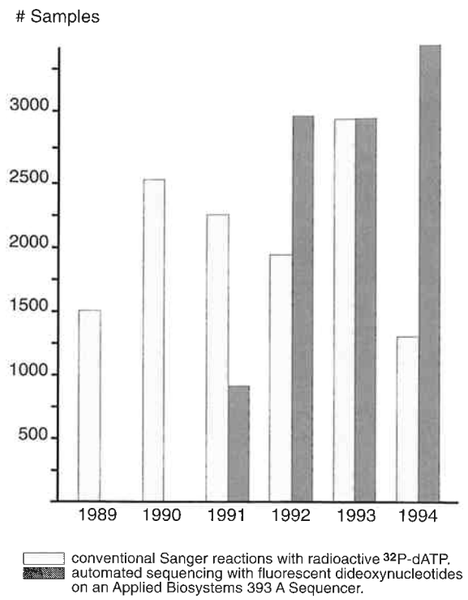

Fig. 3: Total numbers of samples sequenced in 1989 - 1994.

For this reason Robert Kurzbauer did a study to find the most reliable quick procedure for preparing DNA that will give good sequencing results. **Fig. 4** shows that among the „quick and dirty" procedures for isolating DNA the „boiling prep" based on a publication by Holmes and Quigley (1981) gave better results than the widely used „alkaline lysis" protocol (Birnboim, 1983). Robert further looked at the stability of the DNA preparations at 4°C and found increased stability in boiled preps. According to his findings it is highly recommended to follow this procedure, digest with RNAse and then phenolize to increase stability of the DNA sample. The sequencing results obtained are as good as those seen with DNA purified on CsCl gradients or prepared with Quiagen columns. We will continue to do some reactions the conventional way with T7 DNA polymerase and radioactive dATP because sometimes, for reasons not known, the Taq polymerase does not function properly.

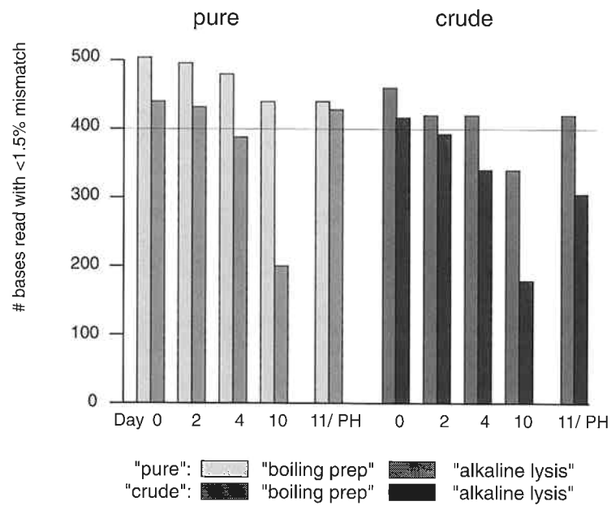

Fig. 4: "boiling prep" vs. "alkaline lysis". pUC 19 was sequenced with M13 universal primer and with DyeDeoxy Taq sequencing kit on a 393 A Sequencer (Perkin-Elmer/ Applied Biosystems). "Pure" DNA is RNAse treated and phenolized immediately after lysis of the *E. coli* strain DH5alpha, "crude" DNA was simply precipitated with ethanol after extraction. Samples were kept at 4°C for the experiment. 11/PH samples were phenol/chloroform treated after 11 days of storage.

Publications during the year

Boehmelt, G., Ulrich, E., Kurzbauer, R., Mellitzer, G., Bird, A. and Zenke, M. (1994). Structure and expression of chicken retinoblastoma gene. *Cell Growth Diff.* **5**, 221-230.

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.

Kenner, L., El-Shabarwai, Y., Hutter, H., Forstner, M., Zatloukal, K., Höfler, G., Preisegger, K.-H., Kurzbauer, R. and Denk, H. (1994). Expression of three- and four-repeat tau isoforms in mouse liver. *Hepathology* **20**, 1086-1089.

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. *Brit. Vet. J.* **151**, in press.

Vorechovsky, I., Kurzbauer, R., Kozmik, Z., Hammarström, L., Smith, C.I.E. and Busslinger, M. (1994). SSCP/*Sac*I polymorphism in the PAX5 gene. *Human Mol. Genetics* **3**, 839.

Other references

Birnboim, H.C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* **100**, 243-255.

Holmes, D.S. and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**, 193-197.

Public Relations Department

Public Relations Officer Heidemarie Hurtl

The Public Relations Department is usually the first contact point for non-scientists wishing to obtain information about the I.M.P. and its research projects. Its services include, among others, the publication and distribution of brochures and booklets, such as the yearly Scientific Report. It is of great importance to the I.M.P. that not only the scientific community but also anyone having an interest in modern science should have the opportunity to find out more about the pursuits and objectives of our institute. To this end, the PR-department organizes guided tours of the institute, where basic techniques in molecular biology are not only explained in theory but, to some extent, may also be observed in the lab. Our guests this year included:

- Eva Faltin (Bundesgymnasium Mödling) with 8th grade students
- Urs Vögeli (Kantonales Laboratorium Basel,Biosafety)
- Helmut Gaugitsch (Umweltbundesamt Wien)
- Eveline Förster (Du Pont Pharma, Bad Homburg)
- Günter Körblein (Boehringer Ingelheim)
- Werner Mair (ORF Wissenschaft)
- Heinz Schuhmacher (Boehringer Ingelheim)
- Walter Reintsch (Boehringer Ingelheim)
- Manfred Pfriemer (Bender Wien)
- Eveline Schütz (ORF Hörfunk)
- Jan Lavicky (Boehringer Ingelheim Prague)
- Peter Placheta (Bender Wien) with 17 senior physicians
- Konrad Ratz (Forschungsförderungsfonds der gewerblichen Wirtschaft)
- Susan Rose (Carrar, Paris)
- Mark Kliss (NASA-Ames Research Center)
- Rolf Krebs (Boehringer Ingelheim)
- Mr. and Mrs. Böhm-Bezing (Deutsche Bank)
- Wolf Renner (Bender Wien)
- Anton Preisinger (Technical University, Vienna)

Gene technology is not only an issue of increasing interest, but also of considerable concern to our society. In 1994, public awareness in Austria has been further stimulated by the new gene technology law which went through a long period of preparation and finally came into effect at the beginning of 1995. Special attention has been drawn to the I.M.P. and the company Bender by the Tumor Vaccine Project (see reports by Max Birnstiel, Matt Cotten and Ernst Wagner). In July 1994, the new production unit for the vaccine was officially opened at Bender and a press conference accompanied the event. Clinical trials on melanoma patients started in December 1994 at Vienna's University Clinic. Since this is Austria's first gene therapy project, it was extensively covered by the media and its progress is being followed with great interest.

The ongoing debate on genetic engineering is a rather emotional one accompanied by strong feelings of hope, but also of fear. The I.M.P. is well aware of its public visibility and the responsibility that comes along with it. We endeavour to contribute to the discussion and to meet the demands of an interested public by keeping our doors open, encouraging visits and, above all, publishing all scientific results.

The role of the PR-department is that of an interface between the scientist and the layperson. Modern molecular biology is a highly complex field and its tools and procedures are not easy to grasp. Mass-media plays an important role in presenting these new and exciting technologies to the public. The PR-department is therefore maintaining close contact to journalists from Austrian newspapers, TV stations and radio stations by arranging visits, interviews and filming sessions. Increasingly, we are also helping out with background information or illustrations for scientific publications in general.

Diploma- and PhD-Theses of I.M.P. Students in 1994

Karoline Briegel (Group Martin Zenke)
"Mechanisms of GATA and erbA transcription factors in red blood cell differentiation." PhD thesis, submitted to the University of Vienna.

Ulrike Burkert (Group Erwin Wagner)
"Studien zur Funktion von Rezeptoren in Stammzelldifferenzierungssystemen der Maus." PhD thesis, submitted to the University of Vienna.

Fatima Cvrckova (Group Kim Nasmyth)
"From Start to Finish: G1 cyclins have a role in yeast cytokinesis." PhD thesis, submitted to the University of Vienna.

Leon Dirick (Group Kim Nasmyth)
"Transcriptional control at the G1-S transition in the yeast *S. cerevisiae*." PhD thesis, FSAGx, Belgium.

Wilfried Ellmeier (Group Andreas Weith)
"Id3: A Dominant Negative Helix-Loop-Helix Gene Isolated from the Human Neuroblastoma Consensus Deletion." PhD thesis, submitted to the University of Vienna.

Birgit Mandl (Group Max Birnstiel)
"Cleavage stage histones of the sea urchin: characterization of a novel set of histone." PhD thesis, submitted to the University of Vienna.

Anne Karina Perl (Group Erwin Wagner)
"Inactivation of the c-*fos* and c-*jun* proto-oncogenes in embryonic stem cells and embryonal carcinoma cells." Diploma thesis, submitted to the University of Vienna.

Awards Granted in 1994

Max L. Birnstiel:
Wilhelm Exner Prize, awarded by the Austrian Gewerbeverein (November 1994)

Leon Dirick:
Schepken's Prize of Genetics, awarded by the Royal Academy of Sciences, Belgium (December 1994)

IMP/Biocenter Seminar List 1994

JANUARY

12.01.94
IGOR VORECHOVSKY (Karolinska Institute, Stockholm)
"Positional cloning of the gene for X-linked agammaglobulinaemia"
Host: Meinrad Busslinger

13.01.94
PAVEL UHRIN (Res. Inst. of Animal Production, Nitra, Slovakia)
"Annexin gene family: structure, function and mechanism of regulation"
Host: Thomas Jenuwein

13.01.94
MICHAEL AGUET (University of Zurich)
"Interferon receptors - Mice lacking in interferon receptors"
Host: Thomas Decker

18.01.94
TILLMAN SCHUSTER (University of Würzburg)
"Search for novel periodically transcribed genes involved in cell cycle regulation"

25.01.94
HEINZ SCHWARZ (MPI, Tübingen)
"Perspectives and limitations in immuno-labelling for light and electron microscopy"
Host: Hartmut Beug

28.01.94
PAUL ORBAN (Vancouver)
"From the autocrine problem to the CRE/LOX system"

FEBRUARY

03.02.94
FRANK GROSVELD (Erasmus Univ., Rotterdam)
"Regulation of the globin gene domain"
Host: Denise Barlow

17.02.94
BRYAN TURNER (Univ. of Birmingham)
"Histone acetylation, transcription and cell memory"
Host: Denise Barlow

24.02.94
IRIS KILLISCH (Univ. of Heidelberg)
"RAB-GTPases on the intersection between the biosynthetic and endocytic pathways"
Host: Björn Neumann

24.02.94
TON ROLINK (Basel Institute of Immunology)
"B-lymphopoiesis in normal and mutant mice"
Host: Meinrad Busslinger

MARCH

01.03.94
GÜNTHER REUTER (Univ. of Halle)
"Genetic and molecular dissection of chromatin in *Drosphila*"
Host: Thomas Jenuwein

03.03.94
KURT NORDSTRÖM (BMC, Uppsala)
"Kinetics of Control of Plasmid Replication by Antisense RNA"
Host: Alexander von Gabain

08.03.94
GERD UTERMANN (Univ. of Innsbruck)
"Molecular genetics of lipoprotein a (Lp a)"
Host: Erwin F. Wagner

10.03.94
JEFFREY W. ALMOND (Univ. of Reading)
"Retargetting, receptor cloning and replication: The three R's of picornavirology"
Host: Ernst Küchler

11.03.94
KLEANTHIS G. XANTHOPOULUS (Center for Biotechnology, Huddinge)
"Transcriptonal regulation in hepatocytes"
Host: Erwin Wagner/Thomas Decker

17.03.94
ULRICH HARTL (Memorial Sloan Kettering Cancer Center, NY)
"The pathway of chaperone-mediated protein folding"
Host: Alexander von Gabain

23.03.94
NANCY H. COLBURN (NIH, Maryland)
"A requirement for AP-1 induction in tumor promotion as modeled in mouse JB6 cells"
Host: Erwin F. Wagner

24.03.94
ANNE EPHRUSSI (EMBL, Heidelberg)
"Germ plasm formation and germline determination in *Drosophila*"
Host: Erwin F. Wagner

25.03.94
IRA HERSKOWITZ (UCSF, California)
"Control of cell cycle progression and cell morphogenesis in budding yeast"
Host: Kim Nasmyth

31.03.94
HANS-HILGER ROPERS (Univ. Hospital Nijmegen)
"X-linked mental retardation: genetic and molecular studies."
Host: Andreas Weith

APRIL

15.04.94
JOACHIM SCHORR (QIAGEN GmbH)
"Large scale purification of Endotoxin-Free plasmid DNA for gene therapy research"

15.04.94
JAN SAP (NYU Medical Center)
"Structure and function of receptor protein tyrosine phosphatases"
Host: Martin Zenke

19.04.94
RENATO PARO (ZMBH, Heidelberg)
"Spreading the silence. Epigenetic mechanisms of transcriptional repression during development."
Host: Thomas Jenuwein

21.04.94
SIDNEY STRICKLAND (SUNY, New York)
"Initiation of Development in Mouse and *Drosophila*"
Host: Denise Barlow

27.04.94
FREDERICK R. SINGER (John Wayne Cancer Inst., Santa Monica, CA)
"Pagets Disease and Osteopetrosis - Basic and Clinical Aspects"
Host: Agi Grigoriadis

28.04.94
RICHARD A. YOUNG (Whitehead Inst., Cambridge)
"A new paradigm for RNA polymerase II transcription initiation"
Host: Kim Nasmyth

MAY

05.05. 94
COLIN BISHOP (INSERM, Marseille)
"The Biology of the Mouse Y Chromosome"
Host: Andreas Weith

10.05.94
GEORG WICK (Univ. Innsbruck)
"Atherosclerosis - an autoimmune disease?"
Host: Erwin F. Wagner

18.05.94
FLORIAN WURM (Genentech, San Francisco)
"Retrotargetting: Efficient vector transfer and stable expression in hamster cells from integrated DNA sequences"
Host: Matt Cotten

18.05.94
TERRY VAN DYKE (Chapel Hill, NC)
"Tumor-suppressor function in cancer"
Hosts: Matt Cotten/Gerhard Christofori

19.05. 94
PIET BORST (The Netherlands Cancer Institute)
"Control of antigenic variation in African trypanosomes: an unusual nucleotide and an unusual transferrin receptor"
Host: Rudolf Schweyen

26.05. 94
GERD JÜRGENS (Univ. München)
"Making a plant seedling - genetic analysis of pattern formation in the *Arabidopsis* embryo"
Host: Dieter Schweizer

30.05.94
TIM STEWART (Genentech, San Francisco)
"The role of interferons in type I diabetes"
Host: Erwin F. Wagner

31.05.94
VARDA ROTTER (Weizman Institute)
"Cancer, the Disease of the Genetic Material"
Host: M.J. Higatsberger

JUNE

06.06.94
ALEXANDRA L. JOYNER (Samuel Lunenfeld Research Institute, Toronto)
"Analysis of mouse *Engrailed* mutants: implications for patterning the nervous system"
Host: Erwin F. Wagner

09.06.94
PETER GRUSS (MPI, Göttingen)
"Functional dissection of mouse developmental control genes"
Host: Erwin F. Wagner

16.06.94
DIETER GALLWITZ (MPI, Göttingen)
"The role of small GTPases in intracellular protein traffic"
Host: Rudolf Schweyen

23.06.94
DON W. CLEVELAND (Johns Hopkins Univ., Baltimore)
"Microtubules, motors and chromosome movement"
Host: Gerhard Wiche

24.06.94
SUZANNE L. TOPALIAN (NIH, Bethesda)
"Specific immune responses to human melanomas"
Host: Max L. Birnstiel

30.06.94
J. RICHARD McINTOSH (Univ. of Colorado)
"Microtubule Dynamics and Chromosome Movement"
Host: Gerhard Wiche

JULY

01.07.94
STANLEY FIELDS (State Univ. of New York)
"The two-hybrid system to detect protein-protein interaction"
Host: Gustav Ammerer

19.07.94
BEAT SCHÄFER (Kinderspital, Zürich)
"Rhabdomyosarcoma cells lack a set of genes expressed during normal myogenesis"
Host: Meinrad Busslinger

20.07.94
RÜDIGER WOSCHOLSKI (ICRF, London)
"Natural and unnatural inhibition of phosphatidylinositol 3-kinase"
Host: Lisa Ballou

AUGUST

03.08.94
JOHN EISMAN (Garvan Institute, Sydney)
"Genetics of Osteoporosis"
Host: Agi Grigoriadis

18.08.94
CLARE HUXLEY (St. Mary's Med. School, London)
"Constructing a mammalian artificial chromosome"
Host: Phd Students (Adam Baker)

25.08.94
FRIEDEMANN KIEFER (OCI, Toronto)
"Searching for genes regulating hematopoietic differentiation"
Host: Erwin F. Wagner

31.08.94
BJÖRN VENNSTRÖM (Stockholm)
"Phenotype of transgenic mice expressing a mutant c-erbA/thyroid hormone receptor"
Host: Martin Zenke

SEPTEMBER

02.09.94
MICHAEL KARIN (UCSD)
"Signal transduction from the cell membrane to the nucleus: Studies on AP-1"
Host: Erwin F. Wagner

05.09.94
DAVID SHORE (Columbia University)
"Silence in the yeast genome"
Host: Gustav Ammerer

06.09.94
PARMJIT JAT (Ludwig Inst., London)
"Molecular genetic approaches toward senesence and immortalization"
Host: Agi Grigoriadis

07.09.94
MARIANO BARBACID (Princeton, NJ)
"The role of the Trk family of receptors in the development of the mammalian nervous system"
Host: Erwin F. Wagner

16.09.94
PETER PFEFFER (HHMI, Salk Inst.)
"Regional specificity of RAR gamma isoforms during *Xenopus* development"
Host: Meinrad Busslinger

22.09.94
ROBB KRUMLAUF (NIMR, London)
"Hox genes and vertebrate development"
Host: Meinrad Busslinger

OCTOBER

03.10.94
JEAN SCHNEIKERT (McGill University, Montreal)
"Characterization and comparison of two mannosidases involved in glycoprotein biosynthesis"
Host: Meinrad Busslinger

10.10.94
DIRK EBERHARD (DKFZ, Heidelberg)
"A TBP-containing multiprotein complex (TIF-IB) mediates transcription specificity of murine RNA polymerase I"
Host: Meinrad Busslinger

11.10.94
IGOR DAWID (NIH, Bethesda)
"The role of the LIM class homeobox gene Xlim-1 in Spemann organizer function"
Host. Max L. Birnstiel

13.10.94
VINCENZO PIRROTTA (University of Geneva)
"Chromatin states, epigenetic maintenance and the regulation of homeotic genes in *Drosophila*"
Host: Erwin F. Wagner

14.10.94
HARALD CREMER (Marseille)
"Phenotypic analysis of N-CAM-deficient mice"
Host: Meinrad Busslinger

25.10.94
SUSAN KANE (City of Hope Nat. Med. Center, CA)
"Multidrug resistance: From molecular biology to gene therapy of cancer"
Host: Karl Kuchler

27.10.94
LUKAS HUBER (Dept. Biochem., Univ. Geneva)
"Molecular mechanisms involved in the generation and maintenance of cellular polarity: From epithelia to neurons"
Host: Hartmut Beug

27.10.94
IAN KERR (ICRF, London)
"JAKs, STATs and signal transduction in response to interferons and other cytokines"
Host: Thomas Decker

NOVEMBER

03.11.94
WALTER KELLER (Biocenter, Basel)
"The biochemistry and genetics of mRNA 3´Processing components"
Host: Gerhard Christofori

09.11.94
CAL HARLEY/MAARTEN LINSKENS (GERON, CA)
"(Im)mortality, telomeres, cell senescence and age-related diseases"
Host: Erwin F.Wagner

10.11.94
ROBERT ROEDER (Rockefeller University, NY)
"Regulation of eukaryotic transcription: General initiation factors, activators and cofactors"
Host: Meinrad Busslinger

11.11.94
KLAUS RAJEWSKY (University of Cologne)
"Targeting mutations into mouse development"
Host: Erwin F. Wagner

15.11.94
RUTH PALMER (ICRF, London)
"The PRK family - a novel PKC Related Kinase family"
Host: Lisa Ballou

17.11.94
PATRICK MATTHIAS (FMI, Basel)
"B-Lymphocyte-specific transcription of immunoglobulin genes: Role of cell-specific activators and coactivators"
Host: Meinrad Busslinger

23.11.94
OLIVIER DANOS (Somatix, Alameda, CA)
"Autologous implants of genetically-modified cells for systemic delivery of therapeutic proteins"
Host: Max L. Birnstiel

24.11.94
CLAUDE DESPLAN (Rockefeller University, New York)
"Homeoproteins and their partners during early *Drosophila* development"
Host: Meinrad Busslinger

28.11.94
KOICHI MATSUO (University of Zürich)
"Methylation and demethylation at CpG sites"
Host: Denise Barlow

DECEMBER

01.12.94
GARY FELSENFELD (NIH, Bethesda)
"Chromatin structure and the regulation of gene expression"
Host: Thomas Jenuwein

12.12.94
ALBRECHT E. SIPPEL (University of Freiburg)
"On the mechanism of cell-type specific transgene expression"
Host: Denise Barlow/Björn Neumann

16.12.94
PIOTR SLONIMSKI (CNRS, Gif-sur-Yvette)
"The yeast genome"
Host: Max L. Birnstiel

I.M.P. Patent Applications 1994

CASE 14/021

Method for treating eukaryotic cells
Inventor: M. Cotten

CASE 14/022

Method for analyzing chromosomal DNA
Inventors: A. Weith, C. Lengauer

CASE 14/023

Method for introducing foreign DNA into higher eukaryotic cells
Inventors: M. Buschle, E. Wagner, W. Zauner

CASE 14/024

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

CASE 14/025

Method for producing and cultivating hematopoetic precursor cells
Inventors: H. Beug, O. Wessely, P. Steinlein, E. Deiner

CASE 14/026

Methods for producing differentiated cells from immature hematopoietic cells
Inventors: M. Zenke, G. Boehmelt, J. Madruga, P. Enrietto

CASE 14/027

Method for introducing DNA into higher eukaryotic cells
Inventors: M. Cotten, T. von Rüden, E. Wagner, K. Zatloukal

CASE 14/028

Method for analyzing the interaction of biomolecules by surface plasmon resonance
Inventors: B. Habermann, P. Steinlein, W. Zauner

CASE 14/029

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

I.M.P. Publications 1994

1. Amon, A., Irniger, S. and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins at the beginning of the next cell cycle. *Cell* **77**, 1037-1050.

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3. Batra, R.K., Wang-Johanning, F., Wagner, E., Garver, R.I. Jr. and Curiel, D.T. (1994). Receptor-mediated gene delivery employing lectin-binding specificity. *Gene Therapy* **1**, 255-260.

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

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8. Busslinger, M. and Bergers, G. (1994). Identification of AP-1-regulated genes. In: *The Fos and Jun families of transcription factors*, P. E. Angel and P. A. Herrlich, eds. (CRC Press, Boca Raton, FL), pp. 133-150.

9. Chen, J., Stewart, V., Spyrou, G., Hilberg, F., Wagner, E. F. and Alt, F.W. (1994). Generation of normal T and B lymphocytes by c-*jun* deficient embryonic stem cells. *Immunity* **1**, 65-72.

10. Cheng, Q., Cant, C.A., Moll, T., Hofer-Warbinek, R., Wagner, E., Birnstiel, M.L., Bach, F.H. and de Martin, R. (1994). NF-kB subunit-specific regulation of the IkBa promoter. *J. Biol. Chem* . **269**, 13551-13557.

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14. Cotten, M., Saltik, M., Kursa, M., Wagner, E., Maass, G. and Birnstiel, M. (1994). Psoralen treatment of adenovirus particles eliminates virus replication and transcription while maintaining the endosomolytic activity of the virus capsid. *Virology* **205**, 254-261.

15. Curiel, T.J., Cook, D.R., Bogedain, C., Jilg, W., Harrison, G.S., Cotten, M., Curiel, D.T. and Wagner, E. (1994). Efficient foreign gene expression in Epstein-Barr virus-transformed human B-cells. *Virology* **198**, 577-585.

16. De Groot, R. P., Ballou, L. M. and Sassone-Corsi, P. (1994). Positive regulation of the cAMP-responsive activator CREM by the p70 S6 kinase: An alternative route to mitogen-induced gene expression. *Cell* **79**, 81-91.

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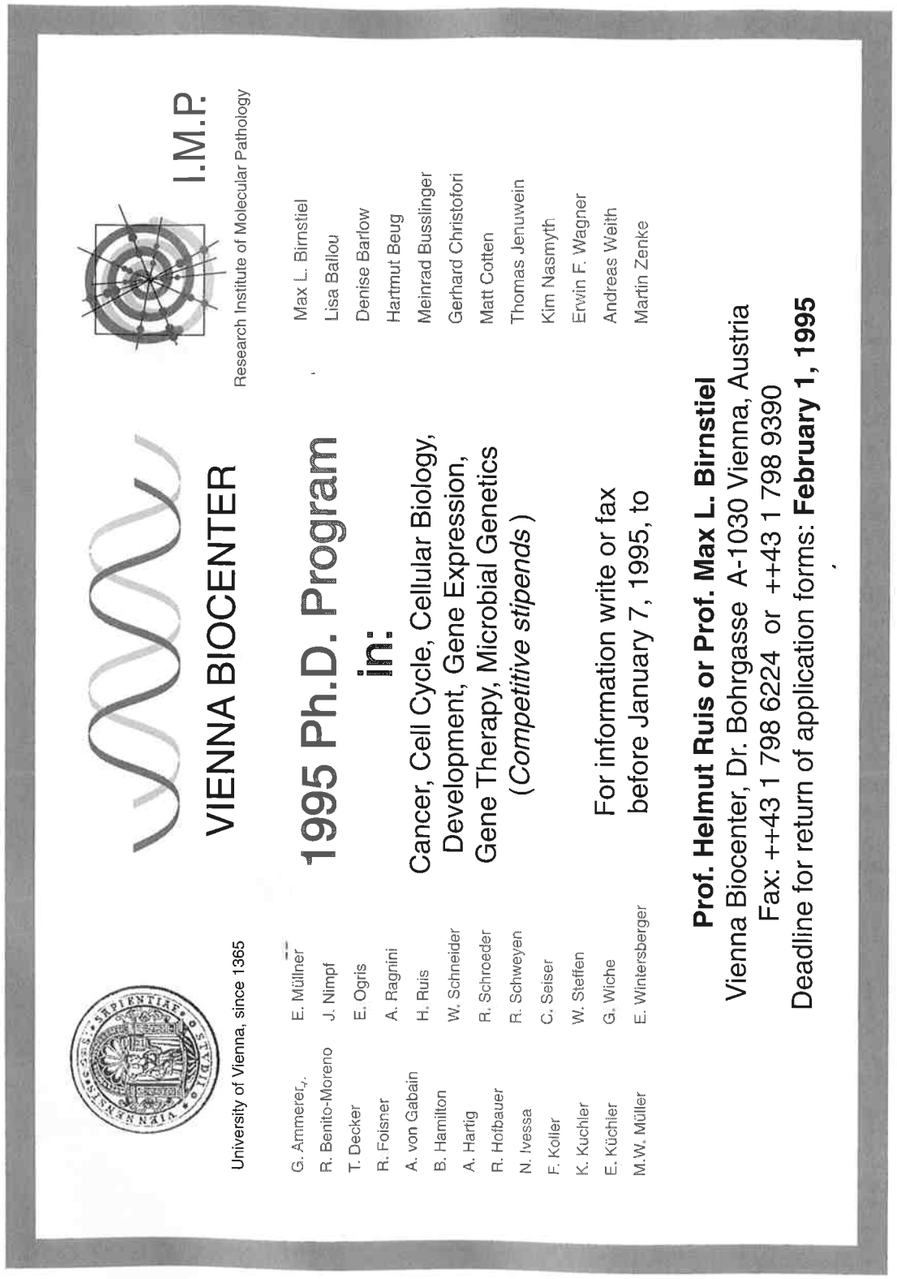

RESEARCH INSTITUTE OF MOLECULAR PATHOLOGY (I.M.P.), VIENNA
5th INTERNATIONAL CONFERENCE ON

"MOLECULAR MECHANISMS OF HUMAN DISEASE"

organized by Meinrad Busslinger, Hartmut Beug, Andreas Weith and Nick Hastie.

to be held at the Austria Center, Vienna
from 13th to 15th May, 1994

This conference aims at presenting recent advances in our understanding, how mutation of specific genes can lead to human disease. Particular focus will be on gene defects in various cancers, in development and in non-neoplastic diseases and on gene therapy.

The following invited speakers have already accepted:

J. Adams (Melbourne)
A. Balmain (Glasgow)
F. Barr (Philadelphia)
A. Berns (Alameda)
G. Christofori (Vienna)
R. Crystal (New York)
O. Danos (Paris)
N. Dyson (Charlestown)
G. Evans (San Diego)
E. Fearon (New Haven)
G. Grosveld (Memphis)
J. Gusella (Charlestown)
N. Hastie (Edinburgh)
P. Herrlich (Karlsruhe)
V. van Heyningen (Edinburgh)
T. Jacks (Cambridge, USA)
D. Lane (Dundee)
J.L. Mandel (Strasbourg)
R. Nicholls (Cleveland)
D. Pardoll (Baltimore)
B. Ponder (Cambridge, UK)
S. Prusiner (S. Francisco)
R. Samulski (Chapel Hill)
U. Siebenlist (Bethesda)
A. Smith (Framingham)
G. Thomas (Paris)
C. Thompson (Chicago)
T. Tlsty (Chapel Hill)
D. Valerio (Rijswijk)
I. Verma (San Diego)

Registration fee: 1.000,- Austrian Schillings (500,- Austrian Schillings for students) This fee will be waived for participants from Eastern Europe.

We cannot provide any financial assistance with travel or lodging

For application forms and further information write to:

Diane Chlup-Turner, IMP
Dr. Bohr-Gasse 7
A- 1030 Vienna, Austria
Tel: +43 / 1 / 797 30 / FAX: +43 / 1 / 7987153

Registration closes: 31st March 1994