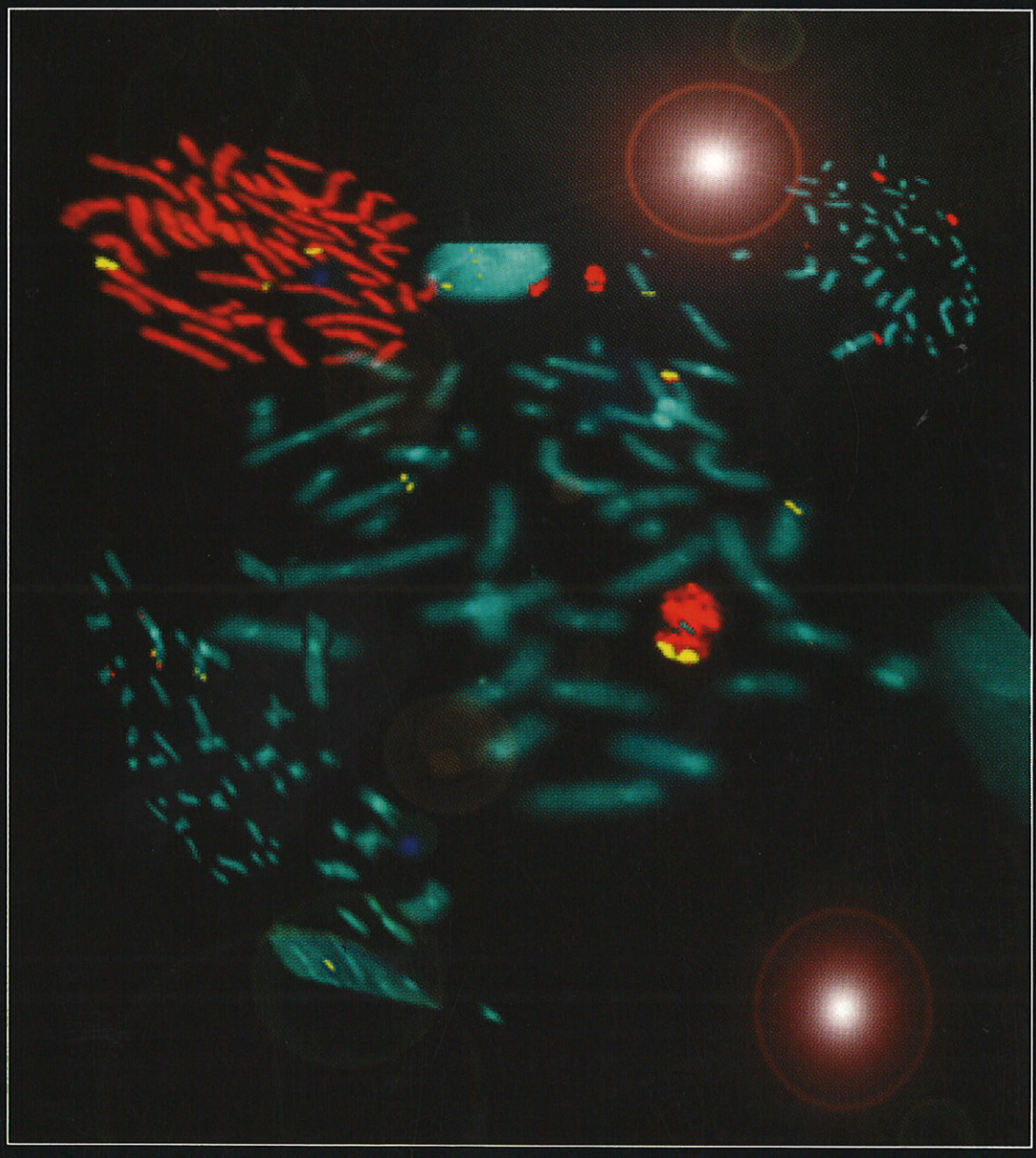
I.M.P. 1992



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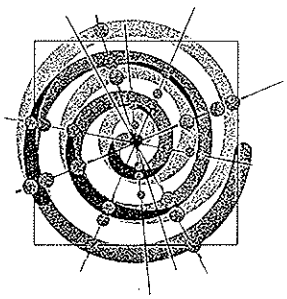

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New methods for gene therapy

Our methods for receptor-mediated transport of DNA into cells have matured over the past year and several areas of application for the technique have been identified. The *in vivo* applications have so far been disappointing, despite many variations in the parameters used, the levels of gene transfer and gene expression reached *in vivo* are too low to be useful for gene therapeutic intervention. However, *ex vivo* transfection of primary cells can be performed with high efficiency. One potential area of interest is the generation of tumour vaccines using the gene transfer technique *ex vivo*. We are further investigating the usefulness of the technique in combatting Hemophilia A and are collaborating with George Dickson (London) on the transfer of the dystrophin gene (the defective gene in muscular dystrophy) into myoblasts.

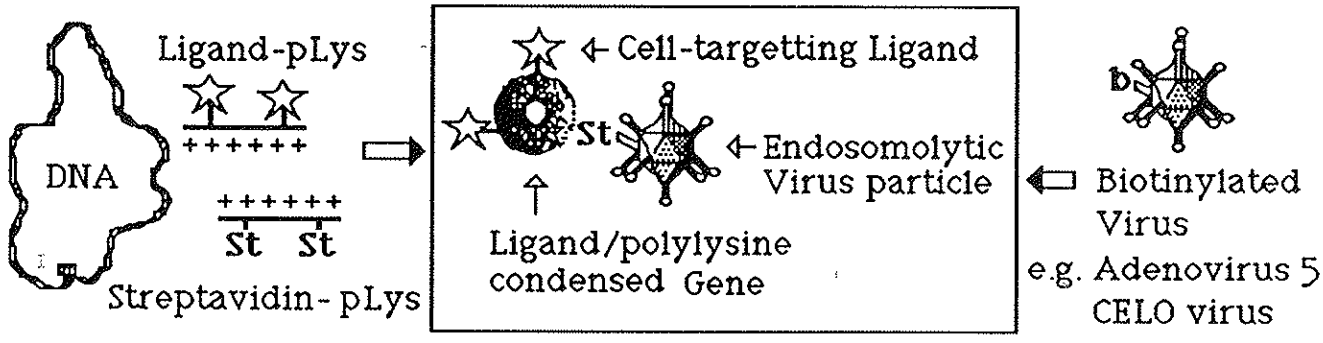
Developmental issues

In collaboration with M. Busslinger (I.M.P.) and E. Dworkin (Bender) we are continuing our analysis of the structure and function of the early cleavage stage histones. Methods have been worked out to knock out the histone H1A mRNA in early embryos, and from these studies a major role of the H1 proteins in the developmental regulation in Xenopus of the oocyte 5S genes seems indicated.

Receptor-mediated gene transfer

Matt Cotten, Kurt Zatloukal, Ernst Wagner,
Mediyha Saltik, Manfred Berger, Helen Kirlappos and
Max L. Birnstiel

We have previously described a DNA transfer system based on receptor-mediated endocytosis. This system uses two components: The gene to be delivered is condensed with a polylysine-ligand into a 100 nm donut linked to the second component of the complex, virus particles containing an endosomolytic activity (e.g. adenovirus). These virus/DNA complexes (see figure) bind target cells via the ligand or via adenovirus receptors, are endocytosed and the lowered pH of the endosome or lysosome triggers a virus endosomolysis activity which allows entry of the material into the cytoplasm (1–4). A variety of modifications and applications of this system for gene therapy have been pursued in the last year.



We have explored the use of an alternative adenovirus type with the hope of identifying a more inert particle which still possesses endosomolytic activity. The chicken adenovirus CELO has been found to possess a more potent, pH-dependent liposome disruption activity than Ad5 and functions as well or better than Ad5 in DNA delivery systems if appropriate means, such as the inclusion of a cell-binding lectin, are taken to ensure uptake by target cells (5). Furthermore, initial toxicity experiments have demonstrated that the chicken adenovirus fulfills many of our requirements for an inert particle possessing endosomolytic activity.

A second important aspect of our gene delivery system is the placement of the cargo DNA on the exterior of the virus particle resulting in a complete separation of the delivered genes from the replication functions of the virus. This allows two types of applications not permissible by normal recombinant viral vectors. **1.** The exterior positioning of the DNA relieves size constraints. We have demonstrated that 48 kb cosmid vectors are delivered with efficiencies comparable to 8-12 kb plasmids (6). We have also successfully delivered to myoblasts, gene constructs encoding the 12 kb dystrophin cDNA which are too large for recombinant retroviral or adenoviral vectors. This may facilitate the correction of dystrophin gene defects in muscular dystrophy patients. **2.** The gene placement outside of the virus means that delivered genes do not participate in the viral replication required to generate recombinant viral vectors. This is useful for the delivery of the clotting factor VIII which contains essential sequences that interfere with retroviral replication. We can demonstrate factor VIII expression in a variety of cells with a myoblast/myotube system displaying the highest levels of expression. This cell/gene combination is being further optimized for *in situ*, muscular delivery of this clotting factor for gene replacement therapy of hemophilia A patients.

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Receptor-mediated gene transfer in vitro and in vivo applications

Kurt Zatloukal, Matt Cotten, Ernst Wagner, Walter Schmidt, Manfred Berger,Mediyha Saltik, Helen Kirlappos, Sonja Ecke and Max L. Birnstiel

We have tested the gene transfer system as described above for its applicability for *in vitro* and *in vivo* gene delivery. A variety of primary cells such as fibroblasts, myoblasts, and endothelial cells are good targets for gene delivery with our system (10%-70% transfected cells without selection). One specific advantage of this method is the ability for large gene-construct delivery (at least 48kb; Ref. 6) and that, in contrast to conventional viral vectors, there is no interference between gene construct and vector. Because of this feature it was possible to express high level of the human factor VIII in different primary cells and cell lines. The highest factor VIII activities (1 Unit/10^6 cells/24 hrs) were found in primary mouse fibroblasts transfected with a factor VIII construct where the B-domain has been deleted. Myoblasts are also suitable target cells for production of factor VIII because they have a high capacity for factor VIII synthesis (almost as high as fibroblasts), they can be obtained by muscle biopsy and reimplanted into the muscle after *in vitro* gene transfer.

Another possible application of the gene transfer system is the generation of cancer vaccines. Tumor cells are genetically modified so that they produce immunostimulatory factors (e.g. cytokines like IL-2, IL-4, IFN-γ, GM-CSF or TNF-α) to elicit a systemic immune response when administered as a vaccine to syngeneic hosts. We have demonstrated the feasibility of this approach using primary human melanoma cells (in collaboration with Georg Stingl, 2nd Clinic of Dermatology, University of Vienna) and a mouse melanoma model as test systems.

Intravenous *in vivo* application of our gene transfer complexes has revealed that certain plasma compounds are inhibitory (serum itself does not interfere). Local application, however, where extensive interaction between plasma and gene transfer components is avoided resulted in successful gene delivery into endothelial cells and liver. In an alternative approach the gene transfer complexes have been delivered into the liver via the bile draining system. This method of delivery allows direct access to the hepatocytes at the bile canaliculus, prevents the interaction with blood constituents and reduces the risk of generating an immune response against the transfection compounds. The total gene expression ($2x10^4$ luciferase light units/mg liver) achieved by this technique, however, is still too low for gene therapeutic application.

In summary, adenovirus augmented, receptor-mediated gene delivery has several advantages over conventional viral vectors and might become an alternative tool for gene therapy, especially when delivery of large gene- constructs is needed, the gene transfer can be performed *ex vivo* and high level of transient gene expression is desired.

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Biological functions of histone H1

Harald Kandolf and Max L. Birnstiel

In addition to its function in sealing the nucleosomal core histone H1 is known to be critical for the formation of higher order chromatin structure. The current understanding suggests that H1 forms the backbone of the 30 nm chromatin fiber, a highly condensed solenoid structure which requires at least loosely positioned nucleosomes. Very likely it is difficult for transacting factors to search for their cognate sequences both outside and inside such a fiber, and around each nucleosome. This accessibility problem raises the important question to what extent the packaging of DNA in chromatin, per se, plays an active role in differential gene activity (1).

One of the best investigated examples of differential gene expression are the *Xenopus laevis* 5S ribosomal RNA genes. During development the large family of oocyte 5S RNA genes (20000 copies/ haploid genome) is selectively repressed and replaced by the almost exclusive expression of the somatic 5S RNA genes (400 copies/ haploid genome). Convincing biochemical evidence has accumulated for a dominant role of histone H1 in this process (2). Our aim was to check the biological relevance of the observations made *in vitro* by direct interference with histone H1 levels *in vivo*. In the pre-gastrula embryo, somatic H1 is clearly under-represented compared to the nucleosomal histones. In eggs and early embryos somatic H1 seems to be functionally replaced by the maternal variant H1m (see report Birgit Mandl). We found by means of quantitative immunoblot analysis that an enormous boost in synthesis at the beginning of gastrulation raises the H1A level to the somatic value of about 1 pg per cell within one hour. Hence the temporal correlation of the first significant appearance of somatic H1 and the almost complete inactivation of oocyte 5S RNA genes nine hours after fertilization appears to be almost perfect.

In our approach we studied the effect of specifically inhibiting the boost in H1A synthesis on oocyte 5S RNA expression. Standard RNA knock out strategies using antisense ODNs have turned out to be not applicable in *Xenopus* embryos due to very low stability and severe unspecific toxicity even at subeffective doses. Hence we have put some effort in improving the tRNA-ribozyme system developed in our lab, which suffered from unsatisfactory activity for efficient in vivo application (3). We found that the ability of tRNA-ribozymes to form a tRNA-like secondary structure is the critical prerequisite for its stability in a cell. The next step was then to express ribozymes as intron-RNA of a *Xenopus* tyrosine tRNA gene. We could demonstrate by injection into oocyte nuclei that extending the size of the natural 13 nt intron to 90 nt did not affect at all the ability of the precursor tRNA to get processed properly. It was shown in vitro that the tRNA secondary structure does not inhibit hybrid formation of tRNA-ribozyme-introns with target RNAs.

For the biological experiments a $tRNA^{tyr}$-ribozyme was constructed targeted to a sequence which occurs twice on a H1A mRNA molecule. Purified in vitro transcripts were microinjected into fertilized *Xenopus* eggs at 50 –100 fold excess over the maternal H1A mRNA pool, which we determined as to be about 160 pg. An immunoblot analysis showed that the H1 synthesis in ribozyme treated gastrula embryos was decreased to 10% of the normal rate. The analysis of ^{32}P-labelled RNAs from blastula and gastrula stages of ribozyme-injected and control embryos was performed using 4 M urea gels, which allow separation of oocyte and somatic 5S RNA (4). It could be clearly demonstrated that the oocyte 5S RNA genes stay very active in gastrula embryos when the significant appearance of H1A protein is prevented, whereas in control embryos these genes get turned off. The specificity of the H1A-ribozyme was confirmed by a control construct which did not show any detectable effect.

We have successfully applied an improved tRNA-ribozyme construct for a specific RNA knock out approach in vivo, solving both the stability and the toxicity problem. Our result provides strong evidence for the biological role of histone H1 in the regulation of a specific gene. We could show that absence of H1 is sufficient to keep oocyte 5S RNA genes in an active state. This finding is in contrast to the current model which explains the developmental regulation mainly with differences in the stability of transcription complexes assembled on oocyte and somatic 5S RNA genes (5). Our observations suggest, that the developmental regulation of 5 S RNA genes in *Xenopus laevis* is rather a consequence of the sequential expression of histones H1m and H1A.

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CSH1 is the first histone H1 in sea urchin development

Birgit Mandl, Meinrad Busslinger and Max L. Birnstiel

CS histones are the first histones to appear in the chromatin of a developing sea urchin embryo. They are synthesized during oogenesis to give a maternal store in the egg and also during the first cell divisions in the cleaving embryo, where they were first detected (CS = cleavage stage). CS histones are responsible for condensing the maternal chromatin in the oocyte and they replace the sperm-specific histones in the male pronucleus after fertilization, thus leading to extensive chromatin remodeling and subsequent activation of the paternal genome (1).

We directed our attention to CS H1 because of the important involvement of histone H1 in higher order chromatin condensation. The CS H1 cDNA as well as its genomic counterpart were isolated from the sea urchin *Psammechinus miliaris*. Sequence analysis revealed that the CS H1 gene belongs to the family of replacement variant histone genes whose expression is independent of U7-snRNP processing and the cell cycle. The CS H1 gene contains an intron and encodes a polyadenylated mRNA with exceptionally long leader and trailer sequences. Northern Blot analysis of *P. miliaris* embryos at different developmental stages showed significant CS H1 mRNA levels up to the 128-cell stage. At this stage excessive transcription from the early H1 gene can already be detected, which leads to rapid dilution of CS H1 protein by the early histone H1 in the chromatin.

CS H1 is the longest H1 protein known. As with all the other H1 histones, it has a tripartite structure with a) a divergent N-terminus, b) a conserved globular core region presumably involved in H1-H1 contacts to give higher order chromatin structures and c) a long, extended and positively charged C-terminus which is believed to mediate binding of H1 to the linker DNA. Sequence comparisons reveal low homology (about 40%) between the CS H1 protein and other sea urchin H1 histones. This was expected since CS H1 resisted all conventional cloning attempts based on heterologous gene probes.

Last year we suggested that the B4 (now: H1m) of *Xenopus laevis* was the vertebrate homologue of CS H1 (B4 being the early embryonic histone H1 in *X. laevis*): Both B4 and CSH1 are transcribed from replacement variant histone genes in a similar developmental pattern to give proteins of comparable sizes which share a 60% homology in their core regions.

Interestingly, there is one feature unique to CS H1: a stretch of acidic amino acids in the N-terminal region whose function is now being investigated.

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The Senior Scientists

IN VITRO MODELS TO STUDY ONCOGENES AND PROTOONCOGENES IN NORMAL DIFFERENTIATION AND ONCOGENENESIS

Hartmut BEUG (Oct. 1988):	Senior Scientist
Marieke von LINDERN (March 1992):	Postdocs
Ernst REICHMANN (March 1989)	
Peter STEINLEIN (January 1990)	
Christian SCHROEDER (until October 1991)	
Irene LEITNER (March 1990):	Students
Oliver WESSELY (May 1991)	
Georg MELLITZER (May 1992)	
Leonie GIBSON (until May 1992):	Technicians
Evi DEINER (October 1990)	
Lisbeth BOER (April 1992)	

Introduction:

In 1992, our group has focused on three main topics. Firstly, we concentrated on elucidating the function of two protooncogenes in normal avian erythroid progenitors. These are c-*erb*A, a nuclear thyroid hormone receptor alpha (TRalpha) and c-*erb*B, an avian homolog of the epidermal growth factor receptor, using mammalian TGFalpha as a ligand (TGFalphaR/c-*erb*B). These genes are the normal counterparts of the two oncogenes (v-*erb*A and v-*erb*B) that are contained in the avian erythroblastosis virus and are responsible for the rapid, fatal erythroleukemia this retrovius causes in chicks.

We had two major reasons to concentrate on protooncogene function in normal cells. Firstly, we found that our previous notion of how v-*erb*A and v-*erb*B transform hematopoietic cells was wrong. Neither are these oncogene *ectopically* expressed in erythroleukemic cells nor do they transform by functioning in an abnormal fashion. Rather, we demonstrated that both protooncogenes (as well as closely related molecules) are expressed in normal erythroid progenitors, that are the target cells of the AEV virus. Furthermore the normal function of these protooncogenes is to regulate or modulate self renewal and differentiation of erythroid progenitors (Figure 1). Thus, understanding protoongene function will help us to explain, how the respective oncogenes function and cooperate in leukemogenesis (Christian Schroeder, Leonie Gibson, Marieke van Lindern and H. Beug in collaboration with Michael J. Hayman, Stonybrook, New York). Due to this focus on protooncogenes, we have done relatively little work on v-*erb*A in 1992. This work will be resumed in collaboration with M. Zenke's group.

In a second main line of research, we try to better understand various aspects of normal and abnormal erythroid differentiation by stably expressing genes of interest in erythroid cells, that can be induced to self renew or differentiate at will. Such cells are obtained by the „conditional oncogene approach". In this approach, erythroid progenitors are induced to proliferate using „transforming" proteins, the function of which can be switched on and off at will. In addition to thermosensitive versions of tyrosine kinase oncoproteins (ts v-*erb*B, ts-v-*sea*.), normal receptor tyrosine kinases (human EGF receptor, avian TGFalpha-receptor/c-erbB) are employed, either overexpressed by a retrovirus or as endogenous proteins normally expressed in certain rare erythroid progenitors. In their „on"-version, [i.e. at the permissive temperature (37°C), or in the presence of the respective ligand] these proteins cause progenitor self renewal, allowing to grow large amounts of progenitor cells. Synchronous terminal differentiation into erythrocytes can then be induced in these progenitor populations by „switching off" (proto) oncoprotein function, either by shift to the nonpermissive temperature (42°C) or by ligand (EGF, TGFalpha) withdrawal.

The types of genes introduced fall into two broad categories. Firstly, a major drawback of the avian hematopoietic cell system is the lack of molecularly cloned hematopoietic growth factors (cytokines), since mammalian cytokines are mostly inactive on avian cells. We have therefore expressed mammalian cytokine receptors (Erythropoietin-(EPO)-receptor, GM-CSF-receptor, Il-3 receptor) in avian erythroblasts to render the cells dependent on cloned mammalian growth factors. This approach has not only been successful in case of the EPO receptor, but now seems to work also in case of the Il-3 receptor and the GM-CSF receptor (Peter Steinlein, Oliver Wessely). We chose the latter two receptors, since cytokines like GM-CSF and Il-3 were so far unknown in the avian system. These receptors will be introduced also into myelomonocytic cells (transformed by a ts mutant of the myb-ets oncogene containing E 26 virus) and into very early hematopoietic progenitors transformed by a conditional v-*rel* oncogene (rendered estradiol dependent by fusing it with the estrogen receptor hormone binding domain (v-*rel*ER; see report of M. Zenke and below)

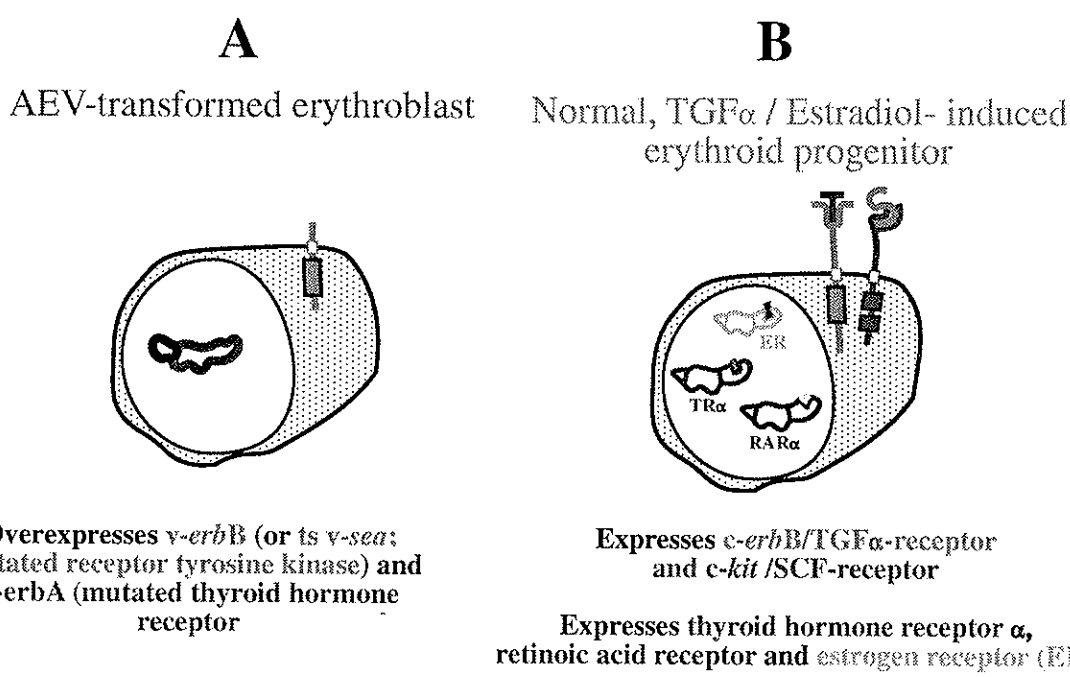

Figure 1. *This simple scheme illustrates our view that transformation of chicken erythroid cells by two oncogenes (the mutated receptor tyrosine kinase v-erbB and the mutated nuclear thyroid hormone receptor v-erbA) (A) is mirrored by regulation of self renewal and differentiation of normal erythroid progenitors by endogenous receptor tyrosine kinases and nuclear hormone receptors (B).*

Transcription factors, that may have a function in hematopoietic cell differentiation are the second group of genes introduced into differentiating hematopoietic cells. Firstly, we employ ligand-dependent transcription factors (estrogen-receptor, retinoic acid receptor) into erythroid cells, concentrating on the estrogen receptor since it seemed to arrest erythroid differentiation in a hormone dependent fashion (Leonie Gibson, Christian Schroeder, Marieke van Lindern). Furthermore, unmodified transcription factors (*spi-1*, *fli-1*, v-*ski*) as well as conditional versions of selected transcription factors are being studied. The latter group comprises ts-mutant proteins (ts-*fos*, ts p53) as well as transcription factors rendered estrogen dependent by fusing them to the estrogen receptor (ER) hormone binding domain (*myc*ER, *jun*ER, v-*rel*ER, *spi-1* ER, *fli-1* ER). Our interest in *fos* and *jun* stems from the fact, that late erythroid differentiation is regulated by a erythroid specific transcription factor (NF-E2) recently cloned in the murine system that binds to DNA elements highly related to the AP-1 consensus sequence and shares structural similarities with *jun* and *fos* (H. Beug and Georg Mellitzer with M. Nicklin and Doug Engel, Chicago). The *spi-1* and *fli-1* oncogenes, on the other hand, play a major role in mammalian erythroleukemia induced by the Friend erythroleukemia virus, as does the p53 gene (Marieke van Lindern and H. Beug, in collaboration with J. Ghysdael, Paris). Finally, the v-*ski* oncogene is of interest, since although being described as a transcription factor mainly involved in muscle differentiation, it nevertheless profoundly affects differentiation of both erythroid and myeloid cells (H. Beug and M.J. Hayman).

The third main topic of the lab is to study how oncogenes act in epithelial cells (Reichmann, et al., 1989). In analogy to our work in hematopoietic cells, we have developed a „conditional oncogene approach" for mammary epithelial cells. This yields *in vitro* epithelial cell culture systems in which processes important for carcinogenesis can be analysed, such as alterations in cell polarity, loss of differentiated functions and changes in epithelial/endothelial or cell-matrix interactions. These are being studied in a mammary epithelial cell system which retains many features of primary mammary gland cells. (E. Reichmann). Again, three hormone-regulated transcription factor-steroid receptor fusion proteins (c-*fos* ER, c-*myc* ER, c-*jun*ER) have been very useful in building up these systems (Ernst Reichmann, Irene Leitner).

A. Protooncogene function in normal erythroid progenitors

C-erbB/TGFaR cooperates with the endogenous estrogen receptor in regulating chicken erythroid progenitor self renewal.

Christian Schroeder, Leonie Gibson and Hartmut Beug

Large amounts (10^{10} to 10^{11}) of normal erythroid progenitors (Pain, et al., 1991) can be grown from normal chick bone marrow by a novel combination of growth-promoting factors. These normal erythroid progenitors express the estrogen receptor (ER) as well as the receptor tyrosine kinase c-*erb*BITGFalphaR. They require both TGFalpha and estradiol for sustained self

renewal *in vitro*, but terminally differentiate upon withdrawal of TGFalpha and inactivation of the ER by antagonist (ICI 164384). Withdrawal of each component alone results in delayed, partial differentiation, suggesting that estrogen and TGFalpha cooperate in maintaining self renewal. To study if the ER alone regulates self renewal, exogenous human ER was overexpressed in erythroblasts devoid of endogenous ER. Upon estradiol activation, the ER efficiently arrested erythroid differentiation and repressed a large group of erythrocyte genes. When similarly overexpressed, ligand-activated c-*erb*B/TGFalphaR caused the reduced expression of a distinct, but overlapping set of genes. The endogenous ER and c-*erb*B/TGFalphaR affected erythrocyte gene expression in a similar, but less pronouced fashion.

Surprisingly, suppression of ER function by antagonist efficiently inhibited erythroblast transformation by tyrosine kinase oncogenes, suggesting a role of the endogenous ER in leukemogenesis. Our current working hypothesis is that the mechanism employed by c-*erb*B/TGFalphaR and ER to regulate normal progenitor self renewal in response to external signals resembles that used by the AEV oncogenes v-*erb*B and v-*erb*A to generate acute erythroleukemia. This hypothesis is currently being studied by trying to identify genes, that are directly regulated by the ER in a hormone-dependent fashion.

Role of the c-erbB/TGFaR and c-kit/SCFR protooncogenes in regulating self renewal and differentiation in erythroid progenitors.

Hartmut Beug, in collaboration with S. Meyer and M. J. Hayman, New York and AMGEN, San Francisco

Following the recent identification by AMGEN of the chicken c-*kit* protooncogene and its ligand, avian stem cell factor (SCF, (Anderson, et al., 1990)), normal erythroid progenitors expressing c-erbB/TGFalphaR and induced to self renew by TGFalpha were found to express c-*kit* and to respond to avian SCF. Analysis of clonal strains of these progenitors showed, that c-*erb*B and c-kit were expressed in the same cells. Stimulation of the 140 kd c-*kit* and 170 kd c-*erb*B proteins with their respective ligands SCF and TGFalpha resulted in their autophosphorylation (Crowe and Hayman, 1991), but no transphosphorylation events were observed.

To our surprise, a second type of normal erythroid progenitor could be grown from normal bone marrow by SCF alone. These progenitors (referred to as SCF progenitors) were much more frequent in normal bone marrow (1 in 500) than those induced by TGFalpha and estradiol (1 in 18,000) and expressed c-*kit* but not c-*erb*B/TGFalphaR. In contrast to the TGFalpha induced erythroblasts (TE-progenitors) the SCF progenitors underwent 6-7 cell divisions and then ceased to proliferate. Withdrawal of SCF from SCF-progenitors resulted in their rapid and complete terminal differentiation into erythrocytes, which was completely dependent on avian erythropoietin (Epo). Interestingly, SCF progenitors differed from TE progenitors also in their ability to self renew. When SCF progenitors were cultivated in presence of SCF and Epo, they were unable to self-renew, but invariably differentiated into erythrocytes. In contrast, TE progenitors were maintained in their self renewing state by either SCF or TGFalpha, when stimulated with these factors in presence of Epo. These results were confirmed using several quantitative assays for erythroid differentiation. They show, that the presence of c-*erb*B/TGFalphaR seems to be required for maintenance of a self-renewing state in erythroid progenitors, suggesting that different types of kinases may have qualitatively different functions in the same cell type (see Figure 2).

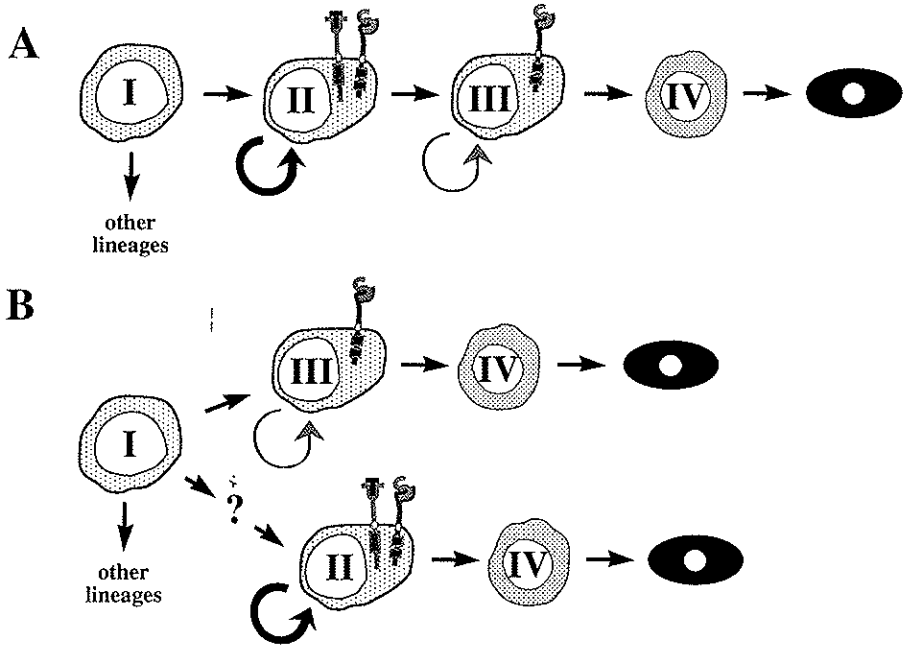

Figure 2. *Hypotheses on the origin and differentiation capacity of SCF- and SCF/TGFalpha-progenitors.*
*This scheme illustrates two possibilities, how SCF/TGFalpha-progenitors [**II**, expressing both c-erbB; (ligand TGFalpha; „T") and c-kit; (ligand SCF; „S")] and SCF-progenitors (**III**, expressing only c-kit) may be interrelated during hematopoiesis. The different ability of SCF/TGFalpha-progenitors and SCF-progenitors to self-renew upon ligand stimulation is indicated by thick and thin circular arrows, respectively.*
*A. Multipotent progenitors (**I**) able to differentiate into other lineages; vertical arrows) give rise to SCF/TGFalpha-progenitors (**II**), which lose c-erbB and develop into SCF progenitors (**III**) before undergoing terminal differentiation and loss of both receptors (**IV**).*
*B. Alternatively, SCF progenitors may directly arise from multipotent progenitors (**I**) and differentiate (**IV**), while SCF/TGFalpha-progenitors represent a specialized progenitor type of unknown origin (arrows with question mark) which differentiates via a parallel pathway not including SCF progenitors.*

Towards identification of the target cells for leukemic transformation by tyrosine kinase oncogenes

Christian Schroeder, Leonie Gibson and Hartmut Beug, in collaboration with M. J. Hayman, New York

The possibility to grow essentially pure cultures of two different types of normal erythroid progenitors (TE- and SCF-progenitors, see above) made it possible to directly analyse, whether one or both types of progenitors were susceptible to transformation (Graf, et al., 1981) by conditional tyrosine kinase oncogenes such as ts-v-*erb*B and ts-v-*sea* or by the proto-onocogene c-*erb*B/TGFalphaR overexpressed from retroviral constructs. Furthermore, it was possible for the first time to analyse, if and how the differentiation phenotype and self-renewal ability of the respective progenitors was altered by oncogenic transformation.

So far, these experiments have been done with TE progenitors. In a first approach, TE cultures were infected with a ts-v-*sea* retrovirus by cocultivation with virus-producing fibroblasts. They were then cloned in semisolid medium lacking TGFalpha and estrogen at the permissive temperature (37°C), thus selecting for transformed cells. As a control, TE progenitors were cocultivated with normal fibroblasts and cloned in Methocel containing TGFalpha and estrogen. Equivalent numbers of clones were obtained from the normal and infected TE progenitors. However, all ts-v-*sea* infected clones grew in absence of TGFalpha, did not respond to this factor in proliferation assays and differentiated into erythrocytes at the permissive temperature, confirming their transformaton by the ts-v-*sea* oncogene. In contrast, all clones grown from the uninfected TE cells were still dependent on TGFalpha and did not differentiate at 42°C. Preliminary experiments suggest, that the ts-v-*sea* transformed TE progenitors are altered in their expression of differentiation antigens as well as in their response to Epo and insulin-like growth factor I (IGF-1). Most clones obtained had also lost their ability to express c-*erb*B/TGFalphaR.

Interestingly, TE progenitors can also be transformed by a retrovirus expressing exogenous c-*erb*B/TGFalphaR, leading to a 20- to 50-fold overexpression of the receptor. These cells are currently characterized for their phenotype.

Currently, a similar series of experiments are done with SCF progenitors. Pilot experiments suggest that these cells are also target cells for transformation by ts-v-*sea* and that the SCF-progenitor-derived transformed cells resemble those obtained from TE progenitors. Future experiments trying to transform TE and SCF progenitors with overexpressed c-*erb*B and c-*kit* proteins will show, whether it is the amount and/or the type of a given receptor tyrosine kinase which determines whether or not an erythroid progenitor is induced to self renew in a sustained fashion.

Tyrosine kinase inhibitors (tyrphostins) as tools to dissect signal transduction pathways involved in self renewal and differentiation of erythroblasts.

Marieke van Lindern, in collaboration with A. Levitzki, Rehovot, Israel

Recently, tyrosine kinase inhibitors (tyrphostins) have been described that exhibit a certain degree of specificity for certain tyrosine kinase types when tested in vitro (Lyall, et al., 1989). Since clear information on a similar specificity *in vivo* was lacking, we tried to identify tyrphostins that would cause specific biological effects in normal and transformed erythroblasts. A useful system for this were TE erythroblasts, since these cells were induced to self renew by stimulation of the endogenous c-*erb*B/TGFalphaR, but induced to differentiate by the combined action of Epo and insulin, both presumably involving tyrosine kinases as well. While seven out of ten compounds tested were inactive or toxic to TE erythroblasts, two tyrphostins were found that specifically inhibited TGFalpha-induced self renewal, while they only marginally affected erythroid differentiation induced by Epo plus insulin.

Evidence is accumulating that several autophosphorylation sites in receptor dimers as well as exogenous substrates may compete for the catalytic domain of tyrosin kinases. In addition, various SH-2 domain-containing proteins bind to specific phosphorylated tyrosin residues in the receptor and thus act in signal transduction. The use of specific tyrosine kinase inhibitors acting on several different defined tyrosine kinases, that have a known cellular function may help to unravel the complex networks through which these kinases might signal.

B. Expression of cytokine receptors and transcription factors in hematopoietic cells transformed by conditional (proto)oncogenes

Function of three mammalian cytokine receptors (muEpoR, hu Il-3R and huGM-CSFR) in avian erythroid and myeloid cells

As outlined above, avian hematopoietic cells transformed by conditional oncogenes are a unique system to study regulation of self renewal and maturation in homogenous populations of hematopoietic progenitor cells. Despite this advantage over comparable mammalian cell systems, avian hematopoietic cells are largely useless to study the function of cytokines (interleukins) in the regulation of hematopoiesis. This is due to the fact, that most mammalian cytokines do not function in avian cells. On the other hand, their avian counterparts are not available in molecularly cloned or purified form (except cMGF, a possible avian homolog to mammalian Il-6; and avian SCF). We have tried to circumvent this problem by expressing mammalian cytokine receptors in avian hematopoietic cells. Besides being able to study the effect of recombinant cytokines on proliferation and differentiation of pure progenitor populations from several lineages, this approach also has the advantage that the cytokine receptors could be analysed for their mechanism of action „in isolation", since growth factors present in foetal calf serum would not act on their avian receptor counterparts.

Murine Epo-Receptor (muEpoR)

Peter Steinlein and Evi Deiner

In 1991, we have expressed the murine Epo-receptor (muEpoR) in erythroblasts transformed by conditional oncogenes like ts-v-*sea* and human c-*erb*B/EGFR, using suitable retrovirus vectors. The exogenous muEpoR was expressed at physiological levels (100-500 receptors/cell and bound ligand with high affinity (Kd = 180-210 pM), suggesting complex formation of the muEpoR with endogenous chicken proteins (see Report 1991). We have now completed analysing the biological effects of the muEpoR and found, that the muEpoR is able to exert all known functions of the endogenous EpoR in mammalian erythroid cells. It promoted survival and terminal differentiation of erythroid progenitors into erythrocytes and cooperated with other growth factor receptors expressed in such progenitors (Insulin-receptor) in a fashion indistinguishable from the endogenous avian EpoR. Furthermore, the muEpoR enhanced the growth rate of oncogene-transformed, self renewing progenitors in an Epo-dependent fashion, suggesting that the signal transduction pathways of oncogenic tyrosine kinases and the EpoR converge at some point. These studies will be continued to eventually understand, why tyrosine kinase oncogenes (v-*erb*B, v-*sea*), but not their normal counterparts (c-*erb*B/TGFalphaR) can bypass Epo receptor function by rendering the transformed cells Epo independent for spontaneous differentiation.

Preliminary experiments suggest, that the muEpoR might also function in a constitutive fashion. Normal, TGFalpha-dependent erythroid progenitors (TE progenitors, see above) infected with a muEpoR expressing retrovirus were invariably induced to differentiate in the presence of TGFalpha plus estrogen, even when EpoR ligand (huEpo) was absent. We are currently analysing the effects of muEpoR on the expression (I) of erythrocyte-specific genes (globins, Band 3, carbonic anhydrase etc.) and (II) of receptors normally expressed in TE progenitors (c-*kit*, c-*erb*B/TGFalphaR, Estrogen-receptor etc.). This might help to answer the question, whether or not EpoR expression might bias a progenitor against self renewal and towards terminal differentiation.

Human Interleukin 3-Receptor (huIl-3-R)

Peter Steinlein, Evi Deiner and Oliver Wessely

Analysis of early avian hematopoiesis is hampered by the fact, that only very few avian growth factors are known and purified. Especially for multilineage growth factors like Interleukin 3 (Il-3) or Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) their existence in the avian system could not even been demonstrated. Neither the use of conditioned media as source for these growth factors was successful in detecting any activity on bone marrow progenitor cells nor where mammalian cytokines active in the avian system. We therefore decided to express the mammalian Il-3 receptor in avian hematopoietic cells, since as reported in 1991 this approach has been quite successful with the murine Epo-Receptor.

In possible contrast to the mammalian Erythropoietin-Receptor (Epo-R), both the IL-3-R and GM-CSF-R consist of at least two polypeptides. Complexes of the Il-3-R and GM-CSF-R alpha chain with a common beta chain are necessary for high affinity binding and biological activity. To express both chains of the Il-3-R in primary avian hematopoietic cells we constructed retroviral vectors with different selection markers which then could be used in combinationwitha transforming avian retrovirus.

To check for correct expression and biological activity of the Il-3-R-complex, we infected avian bone marrow cells with pCRN-DUK-1 (Il-3-R-alpha), pOLI-KH97 (Il-3-R-beta) and ts-v-*sea* as transforming virus. In contrast to infections with viruses containing the muEpoR, numerous ts-v-*sea* transformed, neomycin- plus hygromycin-resistant erythroid clones could be isolated. These clones expressed the expected mRNAs for both Il-3-R chains. Preliminary studies suggest, that several of them are responsive to rhu-Il-3. A >4 fold stimulation of thymidine incorporation was observed at Il 3 concentrations of 5 ng/ml, while half-maximal stimulation occurred at 0.1- 0.2 ng/ml. These studies are being continued with the aim of fully characterizing the function of the mammalian Il-3-R in avian erythroid and myeloid cells.

Human granulocyte macrophage colony stimulating factor receptor (GM-CSFR, alpha chain)

Oliver Wessely, Eva Deiner and Peter Steinlein

After construction of a retrovirus expressing the hu-GM-CSFR alpha-chain from an internal CMV-promoter (see report 1991), this virus was successfully expressed in fibroblasts, ts-v-*erb*B transformed erythroblasts, normal erythroid TE progenitors and myeloid cells transformed by a ts-v-*mybets* oncogene. Receptor was expressed at levels beween 20,000 and 50,000 receptors/cell but (as expected from similar trials in mammalian cells) bound ligand with low affinity (Kd = 2nM). Unexpectedly, a low number of receptors (150 per cell) with higher affinity (Kd = 260 pM) was also detected in transformed erythroblasts, suggesting co-operation with endogenous, avian proteins (beta-chains ?). The fact, that these affinities were clearly lower than reported for human alpha-/human beta-chain complexes suggests, that the human/chicken interspecies complexes are less stable and/or less effective in ligand binding.

To our surprise, the GM-CSF-R exhibited some biological activities even in the absence of an exogenous beta-chain (see above). Firstly, the huGM-CSF-R rendered ts v-*myb/ets* transformed myeloblasts partially dependent on human recombinant GM-CSF, which had no detectable effect on control myeloblasts lacking the endogenous receptor. Furthermore, expression of the GM-CSF-R in TE progenitors and ts-v-*erb*B transformed erythroblasts seemed to have a ligand-independent, constitutive effect. When these cells were induced to differentiate by withdrawal of TGFalpha or temperature shift, respectively, they disintegrated in presence or absence of saturating amounts of chicken Epo, suggesting that GM-CSF-R expression had rendered the cells Epo-unresponsive. One possible explanation for this unexpected phenomenon would be that the GM-CSF competes with the endogenous EpoR for common complexing chains or components of the signal transduction machinery.

We are currently repeating these experiments in cells infected with the GM-CSF alpha chain plus the beta chain presumed to be common to the Il-3-R and the GM-CSF-R. We hope that our experiments may shed some light on the so far elusive question, whether or not different types of cytokine receptors may influence the self renewal/differentiation properties of a given progenitor in a qualitatively different fashion.

Conditional transcription factors as tools to dissect processes in hematopoietic differentiation and transformation

Function of c-mycER, c-junER, c-fosER and v-relER in erythroid cell differentiation

Georg Mellitzer and Hartmut Beug, in collaboration with G. Boehmelt, M. Zenke and M. Nicklin

Since pilot trials to express c-*myc*, c-*fos*, c-*jun* and v-*rel* in erythroid cells have consistently failed, suggesting that overexpression of these transcription factors is incompatible with survival and/or self renewal of erythroblasts (see reports H.Beug and M.Zenke, 1990 and 1991), we plan to repeat these experiments with the respective conditional, estrogen-dependent versions (Eilers, et al., 1989, Superti-Furga, et al., 1991) of these transcription factors (see introduction), trying to completely inactivate their function during erythroblast growth by the effective antagonist ICI 164384. In case of the c-*fos* gene, a ts-*fos* mutant constructed by M. Nicklin is also being used. Respective retroviruses have been constructed and found to function normally in fibroblasts (with the exception of the junER virus, construction of which has still to be completed).

The Friend-erythroleukemia oncogenes spi-1 and fli-1 increase proliferation and retard differentiation of avian erythroid progenitors

Hartmut Beug, with Jacques Ghysdael, Paris

The *spi-1* and *fli-1* oncogenes are transcription factors related to the *ets-1* and *ets-2* protooncogenes. During development of mouse erythroleukemia caused by the Friend erythroleukemia virus, their aberrant expression is induced by a promoter insertion mechanism. To determine, if and how these transcripton factor oncogenes may contribute to erythroleukemia, they were inserted into suitable retrovirus vectors coexpressing a neomycin resistance gene and used to infect TE- and SCF-progenitors (see above) together with the ts-v-*sea* oncogene. Preliminary experiments done to characterise ts-v-*sea* clones expressing *spi-1* or *fli-1* reveal an unusual phenotype. Rather than effectively arresting erythroid differentiation (after ts-v-*sea* inactivation at 42°C) they markedly enhance the growth rate of the differentiating erythroblasts, thus causing a significant delay in differentiation. This effect is particularly pronounced, when the differentiating erythroblasts are stimulated with other growth factors, e.g. avian SCF. Further work employing conditional versions of *spi-1* and *fli-1* (*spi-1* ER, *fli-1*-ER) will hopefully reveal, whether this partial phenotype (that clearly differs from the essentially complete differentiation arrest seen in mouse erythroleukemia cells) is due to inappropriate expression levels of *spi-1* and *fli-1*, and whether it involves altered expression of late erythroid-specific genes or erythroid transcription factors.

The v-ski oncogene cooperates with the v-sea oncogene in leukemic transformation by arresting erythroid differentiation

Hartmut Beug, with Jennifer Larsen and Michael J. Hayman, New York

The avian retrovirus oncogene v-*ski*, a mutated transcription factor involved in muscle differentiation, was analysed for its ability to alter the differentiation program of erythroid cells and to cooperate with tyrosine kinase oncogenes in leukemogenesis. For this, a retrovirus combining v-*ski* with a temperature-sensitive version of the v-*sea* oncogene was constructed. In transformed erythroblasts, v-*ski* disturbed the concerted expression of erythrocyte genes, leading to an abnormal erythroblast phenotype. Expression levels of hemoglobin and erythrocyte anion transporter (band) 3 were elevated, while expression of the erythrocyte-specific histone H5 was strongly suppressed at both mRNA and protein levels. In absence of a functional transforming oncogene (i.e after switching off ts-v-*sea* function at the nonpermissive temperature), v-*ski* repressed or severely retarded temperature-induced erythroid differentiation, again generating an abnormal erythroblast or early reticulocyte phenotype with unusually low histone H5 levels. Thus, v-*ski* cooperates with tyrosine kinase oncogenes in a fashion similar to the v-*erb*A oncogene, although the pattern of genes affected by these two transcription factor oncogenes is totally different.

In chicks, v-*ski* dramatically enhanced the leukemogenicity of the ts-v-*sea* oncogene, leading to massive, mixed erythroid and myeloid leukemia after short latency periods, while ts-v-*sea* alone killed only part of the chickens by inducing anemia. In line with this finding, a number of mixed erythroid/myeloid or even purely myeloid transformed clones could also be obtained from bone marrow transformed with v-*ski*/ts-v-*sea* virus. We are currently trying to characterize these myeloid transformed cells (that morphologically resemble immature granulocytes) and to elucidate, whether v-*ski* alone has any activity in erythroid or myeloid bone marrow progenitors.

p53 and ts p53: Role in hematopoietic cell transformation and immortalization

Hartmut Beug, in collaboration with Eugen Ulrich, Adrian Bird (Edinburgh) and J. Ghysdael (Paris)

In 1990 and 1991, attempts to clarify the role of the p53 protein in immortalization and transformation of fibroblasts and hematopoietic cells were reported. These studies have now been completed (Ulrich, et al., 1992). We employed clones of mortal chicken fibroblasts and erythroblasts transformed by temperature-sensitive v-*src* and v-*erb*B oncogenes as well as immortal cell lines developed from these clones, that retained the conditional transformed phenotype. The expression of p53 and another tumor suppressor gene, the retinoblastoma gene (Rb) was investigated during senescence, crisis and cell line establishment. In ts v-*erb* B erythroblasts and ts v-*src* fibroblasts (as well as v-*myc* macrophages) loss of p53 mRNA or expression of a mutated p53 gene invariably occurred in the early phase of immortalization. In contrast, expression of the Rb gene was unchanged at all stages of immortalization. Inactivation of the original ts oncogene led to loss of the transformed phenotype in fibroblasts and to differentiation in erythroblasts, even in lines that were immortal and lacked p53. The results demonstrate that the process of immortalization is distinct from cell transformation, probably requiring different mutational events.

In order to further analyse possible roles of a normal or mutated p53 in transformation and immortalization, a chicken retrovirus was constructed expressing a ts mutant of the p53 protein. Chicken embryo fibroblasts infected with this virus were found to express high levels of p53 protein. In pilot experiments, we have introduced this ts-p 53 into a continuous, nontransformed chicken fibroblast cell line (CEF 32) and found that the cells proliferate at the nonpermissive temperature (38.5°C) where p53 acts like a transforming, mutant p53. In agreement with data obtained in mammalian cell lines, the ts-p53 expressing CEF 32 cells stopped to proliferate at 34 °C and then disintegrated. The ts-p53 retrovirus may be a useful tool to study both if and how a mutated p53 affects proliferation and differentiation of hematopoietic cells and whether the repression of a normal or mutated p53 in continuous cell lines lacking p53 mRNA (see above) will affect their immortalized state.

C. Conditional oncogenes in normal epithelial cell differentiation and carcinogenesis

Epithelial cell polarity and -differentiation Modulation by conditional versions of early response gene products

Ernst Reichmann, Evi Deiner and Irene Leitner, with M. Busslinger and H. Schwarz, Tübingen

In 1991, we described a novel approach to study the modulation of the polarized phenotype of epthelial cells. This approach involved the expression of c-Fos- and c-Myc-estrogen receptor fusion proteins (c-FosER, c-MycER) in mammary epithelial cells (Superti-Furga, et al., 1991), allowing to activate these hybrid proteins by estrogen for defined time periods and after the cells had achieved their fully polarized organization.

Completing these studies in 1992 yielded the following conclusions. C-Fos-ER or c-MycER in their inactive state (i.e. in the absence of hormone) did not detectably alter the polarized phenotype of epithelial cells. However, when activated by estrogen, these proteins induced distinct and specific changes. Activation of c-Myc-ER deregulated proliferation but did not affect epithelial polarity, as measured by a large number of markers including apically and basolaterally expressed proteins (see below). In contrast, transient activation of c-FosER caused the breakdown of epithelial polarity which, however, was completely reversible after withdrawal of E2. In the absence of E2, Ep-FosER cells formed a regular, ordered monolayer on permeable supports, expressed ZO-1 (tight junction marker), uvomorulin (lateral marker) and DPP IV (apical marker) at the appropriate positions and selectively acidified the basal medium. However, 24 hours after a 30 minute E2-pulse, the cells became round or spindle-shaped and their tendency to pile up was evident. ZO-1 expression was reduced and the circumferential ring of tight junctions had become focally interrupted. Moreover, uvomorulin became expressed basally and at areas of cell-cell contact while DPP IV expression had spread to lateral or even basal sites in many cells. As a further indication of polarity loss, the E2- treated cells acidified both the apical and the basal medium.

Surprisingly, the epithelial monolayer had completely reformed 120 hours after withdrawal of E2. The cells again expressed ZO-1, uvomorulin and DPP IV at the appropriate locations and secreted lactate exclusively into the basal medium, indicating that the cells had completely regained their polar organization. In addition, expression of epithelial-specific proteins was not altered.

Conversely, long-term activation of c-FosER led to the irreversible loss of cell polarity and subsequently to cell conversion into a fibroblastoid phenotype. The cells underwent dramatic changes in their gene expression programme, including the loss or reduction of epithelial-specific markers, and the de novo expression of mesenchymal proteins as well as the gain of expression of certain proteases and their corresponding inhibitors. That this protease expression was of functional significance was shown by our observation, that exogenous protease inhibitors partially inhibited the invasive behaviour of c-Fos-ER-expressing cells in type I collagen gels.

Surprisingly, the addition of hydrocortisone reversed the spindle-shaped, fibroblastoid morphology of the converted c-FosER cells to a clearly epithelial one. Despite these morphological changes, these cells did not attain a fully polarized phenotype. They did not express uvomorulin, which is believed to play a key role in establishing epithelial cell polarity. We currently investigate, whether re-expression of the uvomorulin gene (driven by a retroviral promoter) can restore full epithelial polarity in these cells.

Our findings suggest that immediate early gene products like c-Fos play crucial roles in regulating the plasticity of the epithelial phenotype in both normal and pathological situations (Figure 3).

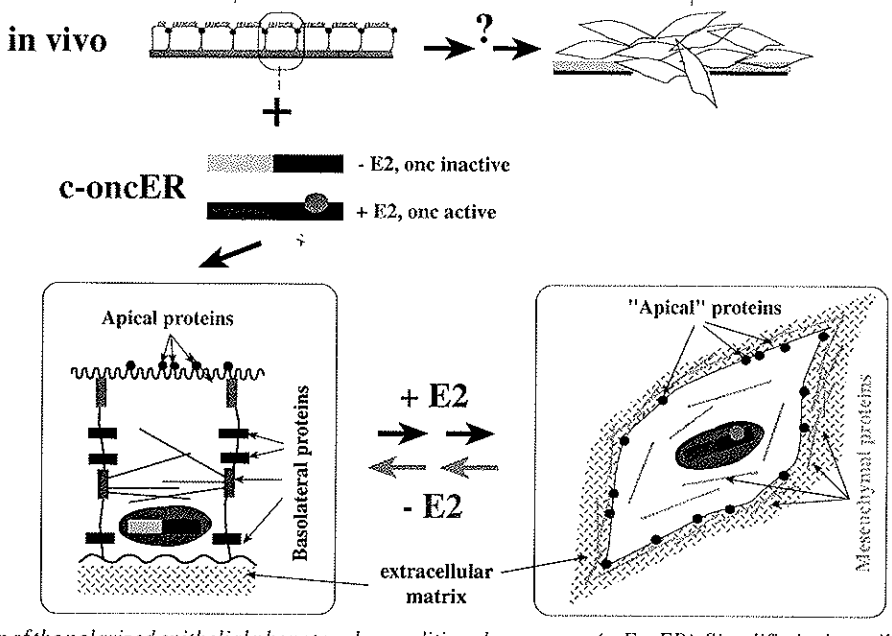

Figure 3. *Modulation of the polarized epithelial phenotype by conditional oncogenes (c-FosER). Simplified scheme illustrating our approach to study the effect on oncogenes on the polarized epithelial phenotype and the types of changes seen with estrogen-activated c-Fos ER.*

Progenitors of mammary gland cell types: Potential target cells for cJunER and other conditional oncogenes

Irene Leitner, Ernst Reichmann and Hartmut Beug, in collaboration with K. Schellander, Wien

In previous work we have established and characterized several spontaneously immortalized, nontumorigenic mouse mammary cell lines (Reichmann et al, 1989), some of which were morphologically heterogeneous, containing three different cell types exhibiting epithelial, myoepithelial and „undifferentiated" cells respectively. Attempts to subclone these cell populations revealed that the majority of initially clearly homogeneous cell colonies with epithelial properties gave rise to heterogeneous cell populations again exhibiting the cell types mentioned above. This indicates that there were epithelial-like precursor cells included in these populations.

To confirm that the cell types present in our populations have a physiological correlate in the animal, we employed antisera against markers of epithelial cells (uvomorulin, ZO-1, DPP-IV, Desmoplakin, cytokeratins), myoepithelial cells (alpha-actin, cytokeratins) and mesenchymal cells (Fibronectin, vimentin). Immunohistochemical analysis on frozen sections from mouse mammary glands of both virgin and midpregnant animals revealed that the antisera stained the expected cells and tissues. *In vivo* we could distinguish the following cell types: Polarized ductal and alveolar luminal epithelial cells, less differentiated epithelial cells surrounding the luminal cells, myoepithelial cells and undifferentiated cap cells located at the very tip of mammary endbuds. Cap cells are the „morphology forming unit" in the developing mammary gland during puberty. They might correlate with the „undifferentiated" cells present in the mixed populations growing out *in vitro*.

Starting from this characterization we asked the question how conditional oncogenes, e.g. chimeras of immediate early transcription factors like Fos and Jun with the hormone binding domain of the estrogen receptor would affect the distinct differentiation stages. We chose the estrogen-dependent c-JunER, since c-Fos ER had already yielded interesting results in epithelial cells (see above, Reichmann et al, 1992). The well characterized cell line LU-Ep1, a spontaneously immortalized, heterogenous line isolated from mammary gland of midpregnant Balb/c mice was infected with a retrovirus expressing c-JunER. Neomycin resistant clones of the different differentiation states were isolated and are presently analysed for estrogen-dependent changes.

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TRANSCRIPTION FACTORS INVOLVED IN DIFFERENTIATION AND SIGNAL TRANSDUCTION

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

Introduction

We have previously identified the B-cell-specific transcription factor BSAP as a mammalian homologue of the sea urchin protein TSAP. TSAP was shown to be responsible for the developmental and tissue-specific regulation of two non-allelic pairs of late histone H2A-2 and H2B-2 genes of the sea urchin (Barberis et al., 1989). BSAP was subsequently detected as a mammalian DNA-binding activity that interacts with the four TSAP-binding sites of these histone gene promoters in a manner indistinguishable from that of the sea urchin protein. Within the hematopoietic system, BSAP is exclusively expressed in the B-lymphoid lineage from the pro-B cell up to the mature B cell stage, while its binding activity is undetectable in terminally differentiated plasma cells (Barberis et al., 1990). BSAP was recently shown to regulate the CD19 gene which codes for a B-lymphoid-specific transmembrane receptor involved in signal transduction (Kozmik et al., 1992). BSAP regulates this gene by binding to the -30 promoter region, as illustrated in Figure 1. The recent biochemical purification and cDNA cloning of BSAP revealed that this transcription factor is a member of the paired domain protein (Pax) family. It is encoded by the Pax-5 gene and has been highly conserved between human and mouse (Adams et al., 1992; Figure 2). Pax proteins are thought to be important regulators in early mammalian development (for review see Gruss and Walther, 1992).

Transcription start sites are shown by short vertical lines and the extent of the BSAP footprint is indicated by a bracket. For details see Kozmik et al. (1992).

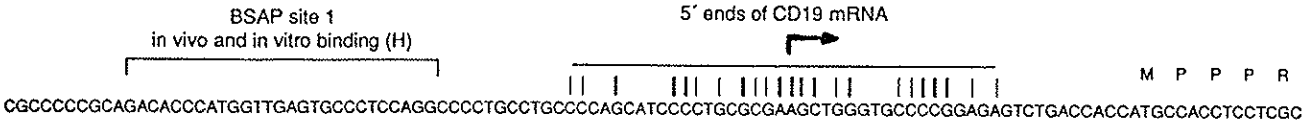

Figure 1. *Interaction of BSAP with the proximal promoter of the human CD19 gene.*

Expression pattern and structure-function analysis of the transcription factor BSAP

Petra Dörfler, Adriano Aguzzi and Pavel Urbánek

BSAP shows a unique spatial and temporal expression pattern in embryonic and adult tissues of the mouse. During embryogenesis, the BSAP gene is transiently expressed in the midbrain and along the neural tube of the central nervous system reaching maximal expression levels around embryonic day 12.5 p.c. (Figure 3). Later, the site of BSAP expression shifts to the fetal liver, where its expression nicely correlates with the onset of B-lymphopoiesis. BSAP expression persists in B-lymphoid tissues into adult life and, starting in the fetal liver, is accompanied by the expression of the BSAP target gene CD19. Unexpectedly, BSAP is also expressed in the testis of the adult mouse suggesting that BSAP may not only play an important role in B-cell differentiation and neural development but also in spermatogenesis (Adams et al., 1992).

In vitro DNA-binding studies with a series of BSAP resection mutants demonstrated that an intact paired domain (Figure 2) is both necessary and sufficient for DNA binding. In order to investigate the transactivation function of BSAP, we have inserted multiple BSAP recognition sequences upstream of the TATA box of a β-globin gene. Transcription from this promoter was strongly stimulated by endogenous BSAP in transfected B-cells and by ectopically expressed BSAP in plasma cells. Preliminary experiments indicate that a peptide consisting of the paired domain lacks any transactivation function although it is transported to the nucleus. The domains responsible for transactivation are currently being mapped in the central and C-terminal part of BSAP.

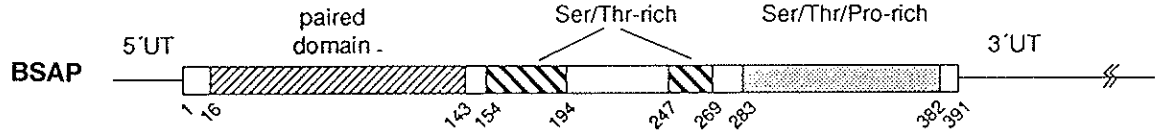

Figure 2. *Schematic diagram of the structural organization of cloned BSAP cDNA. 5'- and 3'-Untranslated sequences (5' UT and 3'UT, respectively) are indicated by a line and the long ORF with its subdomains and corresponding amino acid positions is shown by a boxed region.*

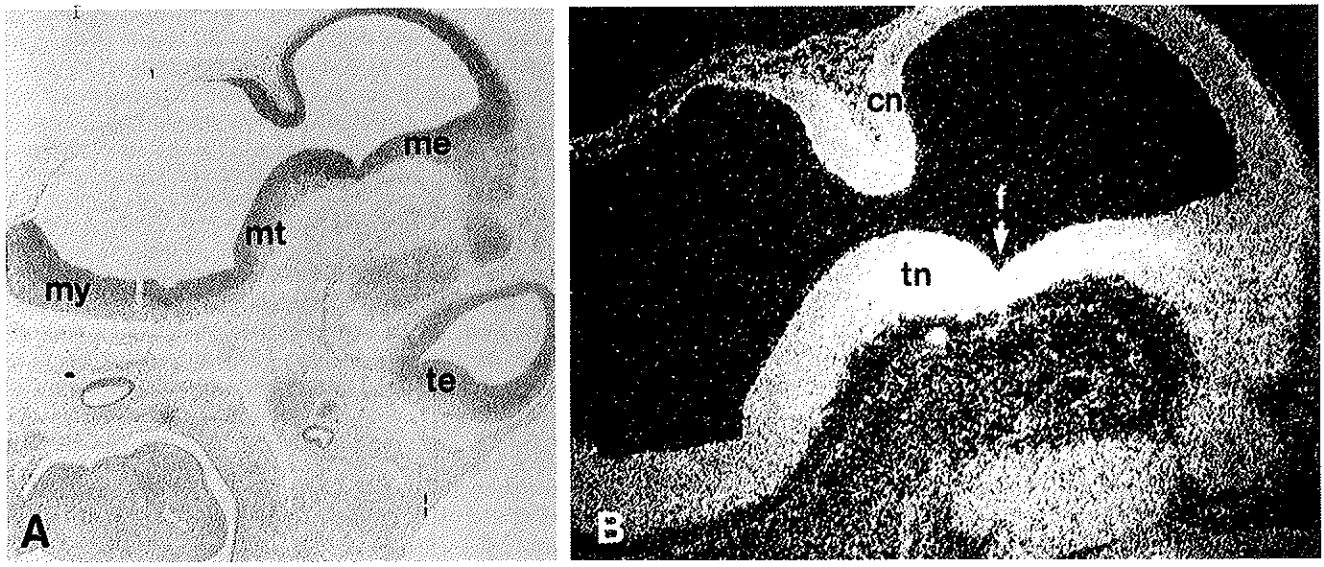

Figure 3. *Expression of the BSAP/Pax-5 gene in the developing brain.*
A) Sagittal section through the brain of an 11.5-day mouse embryo, bright-field image.
B) The same section as in A was hybridized with the antisense BSAP RNA probe and photographed under dark-field illumination. For details see Adams et al. (1992).

Targeted disruption of the mouse Pax-5 gene by homologous recombination in ES cells

Pavel Urbánek in collaboration with Zhao-Qi Wang and Erwin Wagner

As a first step towards understanding the role of Pax-5 in embryonic development, spermatogenesis or B-cell differentiation, we decided to inactivate the gene by homologous recombination in ES cells and to produce a transgenic mouse with a Pax-5 null mutation. Several Pax-5 cosmid clones were isolated from a mouse genomic library and the exon-intron organization and a partial restriction map were determined for the 5' part of the gene. This information was used to design and construct a targeting vector for replacing the first half of the Pax-5 paired box with the E. coli lacZ gene. This construct was introduced into embryonic stem cells and eighty G418-resistant colonies were individually screened by PCR. Six clones with recombination in the Pax-5 gene were obtained and confirmed by genomic Southern blot analysis to be correctly targeted. Three of these clones have been injected into C57Bl/6 blastocysts and several chimeric mice were born which will allow us to study the Pax-5 phenotype in the near future.

Analysis of Pax-8 expression in early development and adult kidney

Zbynek Kozmik

The transcription factor BSAP which is encoded by the Pax-5 gene is most closely related to Pax-2 and Pax-8 and thus all three proteins together constitute a subclass of the Pax family. Pax-8 was previously shown to be expressed in the developing central nervous system, kidney and thyroid gland (Plachov et al., 1990). We have recently subjected the Pax-8 gene to detailled structure-function and expression analyses. By PCR cloning we have identified several alternatively spliced Pax-8 gene transcripts which differ from each other in the central and C-terminal coding regions. The observed splice pattern has been conserved between human and mouse and appears to be regulated during early development of the mouse. However, the alternative splice products differ with regard to their transactivation properties. In addition, an evolutionarily conserved Pax-8 variant was identified, which has lost the potential of binding to known Pax-8 recognition sequences due to the insertion of an additional amino acid residue into its paired domain by alternative splicing.

Characterization of sea urchin Pax proteins

Thomas Czerny

As mentioned in the introduction, TSAP was originally described as a sea urchin transcription factor which binds to each promoter of two nonallelic pairs of histone H2A-2 and H2B-2 genes and which is responsible for their tissue-specific regulation (Barberis et al., 1989). The TSAP-binding sites were subsequently used to identify the mammalian B-cell-specific transcription factor BSAP indicating that both proteins share a highly conserved DNA-binding domain. The recent cDNA cloning of BSAP identified this DNA-binding motif as the paired domain which is characteristic of Pax proteins. We have now developed a general PCR strategy for cloning sea urchin paired domain genes with the aim of identifying and characterizing TSAP cDNA. Three different Pax cDNAs were isolated from sea urchin gastrula embryos. Two of these cDNAs code for proteins with a molecular size, DNA-binding potential and expression profile similar to that of TSAP. Interestingly, only one of the two candidate TSAP proteins shows a high degree of similarity to BSAP in its paired domain.

The third Pax protein is the sea urchin homologue of Pax-6. Genetic lesions in the Pax-6 gene have been associated with the mouse developmental mutant *small eye* and the human disorder *aniridia* (for review see Gruss and Walther, 1992). So far neither target genes nor DNA-binding sites have been identified for Pax-6. As the sea urchin and mammalian Pax-6 proteins have been highly conserved during evolution, we investigated the DNA-binding properties of the sea urchin Pax-6 in detail. Pax-6 and BSAP clearly differ in their DNA sequence recognition, although both proteins share considerable sequence similarity in their paired domains. A series of swaps between the two proteins enabled us to identify three amino acid positions in the paired domain which are responsible for this differential DNA binding.

2) Fos and the regulation of gene expression

Introduction

c-Fos is an essential component of the transcription factor AP-1 and is thought to act as a nuclear mediator of signal transduction by coupling short-term stimulation of the cell to long-term alterations in gene expression. In fibroblasts, Fos/AP-1 has been implicated in the control of cell proliferation and overexpression of c-Fos was shown to result in transformation of these cells. The genes induced by Fos and hence the molecular mechanism of Fos function are, however, still largely unknown. We have established in the past two selective induction systems which allowed us to specifically induce Fos activity into rodent fibroblasts and PC12 pheochromocytoma cells (FosER - Superti-Furga et al., 1991; GalER/Fos - Braselmann et al., 1992a). Taking advantage of these Fos induction systems we identified several Fos-regulated genes. Among them are the genes coding for Fra-1, Fit-1, annexin II, annexin V, ornithine decaboxylase, tyrosine hydroxylase and cytokeratin 18 (Braselmann et al., 1992b; Wrighton and Busslinger, 1992). In the past year we have continued with the characterization of the fra-1 and fit-1 genes.

Characterization of the two Fos-regulated genes fra-1 and fit-1

Gabriele Bergers, Sylvia Braselmann, Paula Graninger

Fra-1 (Fos-related antigen) is an immediate early gene and, like c-*fos*, belongs to the AP-1 gene family. The fra-1 gene is also induced by v-Fos and FosB indicating that it is under the control of AP-1 activity in general. The AP-1-induced increase of fra-1 mRNA synthesis is a general phenomenon, as it is also observed in cell types (PC12 cells and osteoblasts) other than fibroblasts. Fra-1 gene induction is mediated by cis-acting enhancer sequences which have been mapped between position -2000 to -3700 upstream of the fra-1 gene.

So far we can only speculate about the significance of fra-1 up-regulation by c-Fos/AP-1. Fra-1 is a more weakly transforming oncoprotein than c-Fos, because overexpression of Fra-1 in rat fibroblasts promotes only growth in soft agar, but does not result in morphological transformation. Furthermore, Fra-1 appears to lack any transactivation function, as fusion proteins between the DNA-binding domain of Gal4 and Fra-1 are unable to transactivate a Gal4 -responsive promoter in contrast to the corresponding Fos fusion proteins. We therefore speculate that Fra-1 is involved in the down-modulation of Fos activity in response to signal transduction.

Fit-1 was identified as a Fos-induced transcript in rat fibroblasts by differential cDNA cloning. Its gene was shown to be the rat homologue of the mouse T1 gene, a member of the immunoglobulin gene superfamily. Nuclear run-on analyses demonstrated direct transcriptional regulation of the fit-1 gene by Fos-ER. However, analysis of the fit-1 gene failed to identify a Fos-responsive element in the first 800 bp of its promoter as well as in the first intron suggesting that the regulatory sequences mediating the Fos effect reside farther upstream or downstream. Fit-1 is a secreted protein which has no apparent morphological effects on fibroblasts. It may, however, be involved as a paracrine growth factor in the communication of fibroblasts with other cell types.

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CELL CYCLE CONTROLL IN YEAST

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Cell cycle control in yeast and other eukaryotes

Kim Nasmyth

Unlike early embryonic cleavage divisions in certain animals, cell cycle progression in yeast and probably also in all metazoan somatic cells requires the periodic transcriptional activation of certain key genes. Thus far, the only clear examples are genes that encode a class of unstable „cyclin" proteins, which bind and activate the cdc2/CDC28 protein kinase the G1-specific cyclins encoded by *CLN1, 2, 3*; a B-type cyclin implicated in DNA replication encoded by *CLB5*; and four B-type cyclins inolved in mitosis encoded by *CLB1, 2, 3, 4*. *CLN1*, *CLN2*, and *CLB5* are transcribed in late G1, as cells undergo START. A transcription factor composed of Swi4 and Swi6 proteins activates *CLN1* and *CLN2* transcription via a positive feedback loop in which Cln proteins activate their own transcription. Swi4 contains a site specific DNA binding domain whereas Swi6 is mainly necessary for regulating transcription. A different but related transcription factor composed of a 120 kd protein (p120) complexed to Swi6 is responsible for the late G1-specific transcription of most DNA replication genes including *CLB5*. Repression of genes activated by Swi4/Swi6 but not those regulated by p120/Swi6 requires mitotic cyclin activity. Thus, G1 cyclins activate their own synthesis and subsequently that of G2 cyclins, which then repress the synthesis of G1 cyclins.

Mitotic cyclins are required for repression of the G1 cyclins in yeast

Angelika Amon and Kim Nasmyth

In the budding yeast *S. cerevisiae* the protein kinase $p34^{CDC28}$ is required for passage throught START and for progression through mitosis. The specificity of the p34 kinase is determined by its regulatory subunits called cyclins. At the G1/S transition $p34^{CDC28}$ is associated with the G1 cyclins to form the G1 kinase and during mitosis it is complexed with the mitotic cylcins CLB1, 2, 3 and 4 to form the mitotic kinase. To determine which processes during the cell cycle require the mitotic kinase, we constructed a yeast strain with a conditional mitotic kinase. To this end, temperature sensitive (ts) mutants in one of the mitotic cyclins CLB2 were generated. Strains carrying a *clb2-ts* allele as the sole mitotic cyclin arrest at the restrictive temperature as budded cells with a 2N DNA content. However, these cells fail to form a mitotic spindle, indicating that the mitotic kinase is required for generation of the mitotic spindle but is not necessary for DNA synthesis or bud formation.

The G1 kinase and the mitotic kinase are active in different stages of the cell cycle. G1 kinase activity is maximal at the G1/S transition, disappearing soon thereafter while the mitotic kinase is activated as the G1 kinase activity declines and peaks in metaphase. To further our understanding of the oscillation of the two forms of the p34 CDC28 kinase, we have asked whether the mitotic kinase is required to turn off the G1 kinase. Since expression of the G1 and mitotic cyclin RNAs correlates with the presence of the corresponding kinase, we analysed the G1 cyclin RNA levels in a strain with a temperature sensitive mitotic kinase. We found that this strain fails to turn off G1 cyclin transcription as well as the G1 kinase at the restrictive temperature, indicating that the mitotic kinase is required for repression of the G1 kinase. Another set of genes encoding DNA replication enzymes is transcribed at the same time during the cell cycle as the G1 cyclins. Surprisingly, repression of these genes does not require the mitotic kinase indicating that the effect of the mitotic kinase on the G1 cyclins is specific. Further studies to determine the targets of this repression are in progress.

The role of MAP kinase-like proteins in pheromone dependent cell cycle arrest in S. cerevisiae

Anton Gartner, Beverly Errede, Kim Nasmyth and Gustav Ammerer

Pheromone induced cell cycle arrest and induction of differentiation in S. cerevisiae resembles negative growth factor action in mammalian systems. Genetic data suggested that pheromone-dependent signal transduction requires the action of several protein kinases including Ste11, Ste7 and Fus3. Fus3 is a kinase with similarity to mammalian MAP kinases.

We have shown that Fus3 is activated *in vivo* in response to pheromone by Ste7 dependent phosphorylation on threonine180 and tyrosine182. *In vitro* immunoprecipitated Ste7 kinase is able to phosphorylate bacterially-produced Fus3 at the appropriate sites thereby activating its kinase avtivity. These data strongly suggest that Ste7 is the physiological fus3 activator. Recent evidence showing that MAP kinase activators from different organisms are related to Ste7 indicates that homologous signal transduction pathways may be conserved in evolution.

In collaboration with the lab of I. Herskowitz, we analysed the phosphorylation of the Far1 protein by Fus3. Far1 has been shown to be required for the down-regulation of G1 cyclin activity. Our current working hypothesis is that Fus3 phosphorlyates Far1, thereby potentiating its G1 arrest activity.

CLB5: a new cyclin gene involved in DNA replication in yeast

Etienne Schwob and Kim Nasmyth

The protein kinase $p34^{cdc2}$ is the major trigger for cell cycle progression in all eukaryotes. In *Saccharomyces cerevisiae*, this kinase which is encoded by the *CDC28* gene is required both for START (G1/S boundary) and progression through mitosis. Its activity is regulated by the association to the G1 cyclins *CLN1,2,3* or mitotic B-type cyclins *CLB1,2,3,4* and deletion of these cyclins lead to G1 or G2 arrest, respectively.

We have discovered a fifth B-type cyclin gene, *CLB5*, which is adjacent to *CLB2* and has a major role in S-phase progression. Indeed, deletion of the *CLB5* gene, while not lethal, leads to a dramatic increase of the population of cells with a DNA content between 1N and 2N. Consistent with a role in DNA synthesis, *CLB5* is expressed early in the cell cycle like DNA replication genes, under the control of the p120 and *SWI6* proteins binding to MCB elements in its promoter. When overexpressed, *CLB5* can rescue a *CLN1,2,3* deletion indicating an overlapping role at the G1/S transition. Strains deleted for *CLB3,4* and *5* are not viable, suggesting that these three cyclins act in combination to perform some steps in DNA synthesis. *CLB5* overexpression suppresses the *cdc28-13* thermosensitivity and therefore probably binds to $p34^{CDC28}$. *CLB5* is the first yeast cyclin showing a marked effect on S-phase progression. The growing number of discovered cyclins suggests that $p34^{CDC28}$ may control each step of cell-cycle progression by forming complexes that are targeted to specific subsets of substrates. Further studies to analyse the role of *CLB5* in DNA synthesis and to identify substrates of the *CLB5*-associated kinase are in progress.

A complex between SWI6 and a 120KD protein regulates genes involved in DNA replication in yeast

Thomas Moll, Christian Koch, Léon Dirick and Kim Nasmyth

Yeast cells at a certain point in their progression through G1 become restricted to mitotic cell division, a stage of the cell cycle that has been named START. Once past this step, a number of genes are activated, including the *HO* enconuclease, *CLN1, CLN2* and many of the genes involved in DNA replication. When analysing their promoters, two related DNA motifs, called SCBs (swi cell cycle box; $CACGA_4$) and MCBs (MluI cell cycle box; ACGCGTNA), were identified as being responsible for START-dependent transcriptional activation. SCBs have been found in the *HO* as well as in the *CLN1* and *CLN2* promoters and a transcription factor complex containing the SWI4 and SWI6 proteins was shown to bind to this element and at least in the case of the *HO* and *CLN2* genes shown to be responsible for their START-dependent activation.

The promoters of the DNA replication genes all contain one or more MCBs and we have recently demonstrated not only that START-deendence and cell cycle regulation of transcription are dependent on *SWI6 in vivo*, but also that SWI6, together with a 120kD protein (p120) distinct from SWI4, binds to this MCB motif *in vitro*. The p120/SWI6 protein complex has been purified to near homogeniety from yeast and recently microgram quantities of pure protein were obtained. Antibodies against p120 are being raised.

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GENE FUNCTION IN MAMMALIAN DEVELOPMENT AND ONCOGENESIS

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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 oncogenesis. Although the function of genes is exerted at the cellular level, all our approaches are aimed at studying the role of specific gene products in the context of the intact animal. 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 in-to hematopoietic stem cells of bone marrow-reconstituted mice.

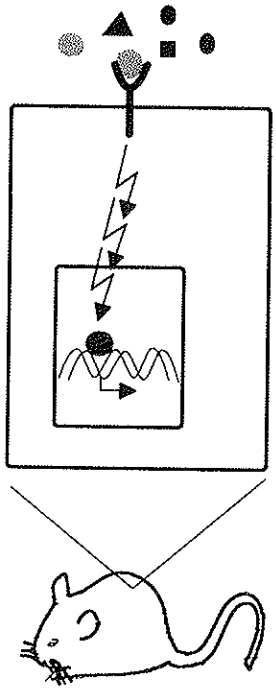

Fig. 1 Outline of research projects

I. ANALYSIS OF GENE FUNCTION BY HOMOLOGOUS RECOMBINATION IN ES CELLS AND MICE

During 1992 we have successfully established the „routine" generation of mice with pre-determined genetic changes using homologous recombination in ES cells. Our main focus lies in unravelling the function of AP-1 *in vivo* (see below). In addition, we have generated mice lacking the neural cell adhesion molecule AMOG (in collaboration with Melitta Schachner, Zürich) and the ADP-phosphoribosyl transferase gene (ADPRT; in collaboration with Bernhard Auer, Innsbruck). Furthermore, we are in the process of generating mice lacking the B-cell specific transcription factor, PAX-5 (see report by Meinrad Busslinger).

Function of transcription factor AP-1 in vivo

Zhao-Qi Wang, Frank Hilberg, Agi Grigoriadis, Uta Möhle-Steinlein
and Jun Liang, Karl Schellander, University of Vienna

The major components of the AP-1 family of regulatory proteins are the products of the proto-oncogenes c-*fos* and c-*jun*, both thought to be of central importance in maintaining normal cellular growth and differentiation. To dissect the functional relevance of AP-1 proteins *in vivo*, ES cells were used to inactivate the c-*fos* and c-*jun* genes.

ES cells lacking Fos were viable and showed no obvious abnormalities with respect to morphology, growth rate, differentiation potential *in vitro* and expression of other *fos*/*jun*-related genes. Mice heterozygous at the c-*fos* locus appear normal whereas homozygous *fos* -/- mice develop multiple defects shortly after birth. The mutant mice are severely growth retarded as early as two weeks of age and exhibit deficiencies in bone remodeling and in tooth eruption, which are reminiscent of osteopetrosis (Fig. 2). Growth plate abnormalities were also observed suggesting additional alterations in endochondral bone formation. Moreover, hematopoiesis is perturbed in mutant mice as evidenced by a 50-80% reduction in the number of B and T cells. In contrast, myeloid lineages do not seem to be affected. In addition, we observed a transmission distortion through the heterozygote females. These results are significant in view of the known expression profile of the endogenous c-*fos* gene, and the results obtained by c-*fos* overexpression in transgenic and ES chimaeric mice, emphasizing an essential function of Fos in osteogenic and hematopoietic cell development.

When both c-*jun* alleles were inactivated in ES cells, no effects were observed on growth, differentiation and AP-1 activity *in vitro*. However, these cells were drastically impaired in their ability to function *in vivo*, e.g. in tumor formation following subcutaneous injection into syngeneic mice. Experiments aimed at generating Jun-negative mice suggest that homozygous *jun* -/- mice die during development at midgestation. A detailed analysis on embryos lacking c-Jun is currently being carried out.

Taken together, these results support the notion that the members of the AP-1 family of transcription factors have different and specific functions *in vivo*: Whereas functional c-Jun protein appears to be important at critical points during embryogenesis, the function of Fos is restricted to specific cellular compartments. To better define the role of AP-1, we are presently inactivating other AP-1 genes such as *fra*-1 and *fos*B.

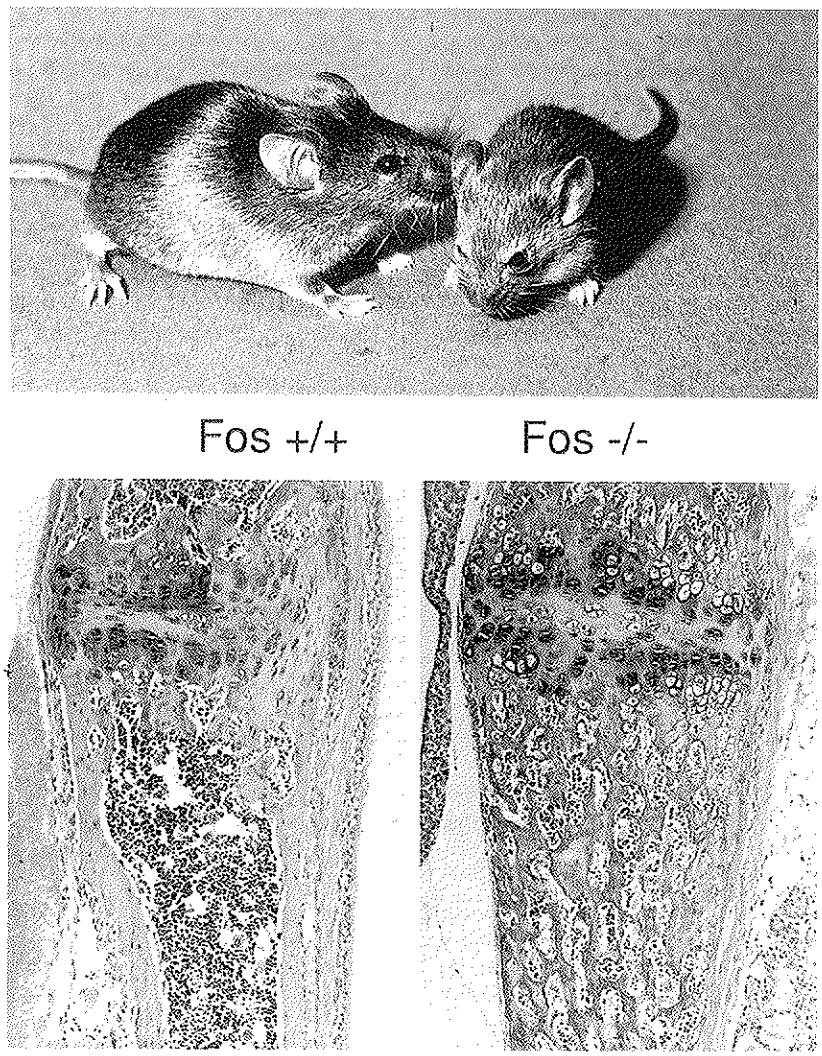

Fig. 2 Phenotype of mice lacking c-Fos. Note the growth retardation and the osteopetrotic bone in the Fos -/- mouse.

Analysis of target cell specificity and AP-1 function in osteogenic cell lines

Transgenic mice expressing c-*fos* under the control of the murine H2-K^b class I MHC promoter develop osteosarcomas with 100% penetrance and the transgene is expressed in bone cells prior to the onset of tumor formation. To investigate further the target cell specificity and the potential genes which may be affected by Fos we have analyzed the expression of AP-1- and bone-associated genes in primary and clonal tumor-derived cell lines.

All primary cell lines isolated from c-*fos* transgenic tumors expressed high levels of exogenous c-*fos* and some formed osteosarcomas *in vivo*. RNA *in situ* hybridization using a probe specific for the transgene and immunocytochemistry for Fos protein confirmed that the osteogenic cells expressed high levels of exogenous c-*fos*. Expression of other AP-1 genes showed that while the *jun* family of oncogenes (c-*jun*, *jun*B, *jun*D) were expressed at moderate to high levels, *fra*-1 levels appeared to be correlated with levels of exogenous c-*fos*. All osteoblastic marker genes tested were expressed in all cell lines with the exception of osteocalcin, which was low or undetectable in cells expressing exogenous c-*fos*.

To further investigate the nature of the target cell populations, we cloned two cell lines and the properties of 12 single clones were assessed. All cells expressed high levels of exogenous c-*fos* as well as c-*jun* and *fra*-1. All clonal cell lines were tumorigenic and one clone, designated P1.15, retained the ability to differentiate into bone *in vivo*. Expression of osteoblastic genes, especially alkaline phosphatase and osteocalcin, varied between the clones. In order to investigate whether the activities of the different *fos*- and *jun*-related genes are linked in a functional way to the observed variation in tumorigenicity and gene expression, we are currently analyzing AP-1 binding activity in nuclear extracts from different representative cell clones.

II. MECHANISM OF MIDDLE T-INDUCED ONCOGENESIS

Friedemann Kiefer, Ingrid Anhauser and Adriano Aguzzi

Chimeric mice generated by blastocyst injection of ES cells expressing the mouse polyoma virus mT oncogene (PymT) die at midgestation from multiple endothelial tumors called hemangiomas. Endothelial cell lines (End.cells) can be readily derived from these primary lesions as well as from hemangiomas that form in newborn mice after infection with a PymT-transducing retrovirus. To better understand how PymT oncogene exerts its specific effect on endothelial cells a number of different *in vivo* and *in vitro* approaches are being employed.

PymT has been shown to bind and activate the src family tyrosine kinases pp60$^{c\text{-}src}$, p59^{fyn} and pp62$^{c\text{-}yes}$. In collaboration with Phil Soriano, Houston, we could demonstrate that hemangiomas are efficiently formed in c-*src* and c-*fyn*-deficient newborn mice following infection with a PymT-transducing retrovirus. Transformed endothelial cell lines were readily derived from these lesions. Preliminary results suggest that hemangiomas can also arise in c-*yes*-deficient mice after virus injection, suggesting that the three tyrosine kinases can substitute for each other. Furthermore, the homologous mT oncogene of the hamster polyomavirus (HamT), which binds to pp59^{fyn} but not to pp60$^{c\text{-}src}$, also causes hemangiomas in mice, albeit at a significantly lower frequency and with a longer latency period than the mouse PymT (in collaboration with Sara Courtneidge, EMBL).

To investigate the pronounced specificity of PymT for endothelial cells as well as the difference between PymT- and HamT-mediated transformation, we are taking advantage of a technique that allows the generation of completely ES cell derived fetuses (see below). Using this technique PymT- and HamT-expressing ES cell lines are used to understand the action of these two oncogenes on early stages of mouse development. Preliminary data suggest that PymT-expressing ES cells can form all structures of the embryo proper.

Generation of "ES-mice"

Friedemann Kiefer

In contrast to ES chimeras generated by blastocyst injection, completely ES-derived mice can be generated following aggregation of ES cells with tetraploid host embryos (Nagy et al. Development, **110**, 815 - 821, 1990). The tetraploid embryos are produced by electrofusion of 2-cell stage embryos which are allowed to develop to morulae *in vitro* before aggregation with a small number of ES cells (Fig. 3). Following blastocysts transfer, these aggregates have the potential to develop into completely ES-derived fetuses and mice, since the tetraploid cells only contribute to the formation of extra-embryonic structures (Fig. 3).

We have successfully established this novel approach in our laboratory and have obtained ES-derived fetuses (with D3 ES cells) and mice with the recently characterized ES cell line R1 (kindly provided by Andras Nagy). Presently we are using this powerful technique to study mT-induced oncogenesis (see above), but envisage a broad application in the future for analyzing gene function in hematopoietic development, for gene expression analysis as well as for shortening the time to generate mice with predetermined genetic changes.

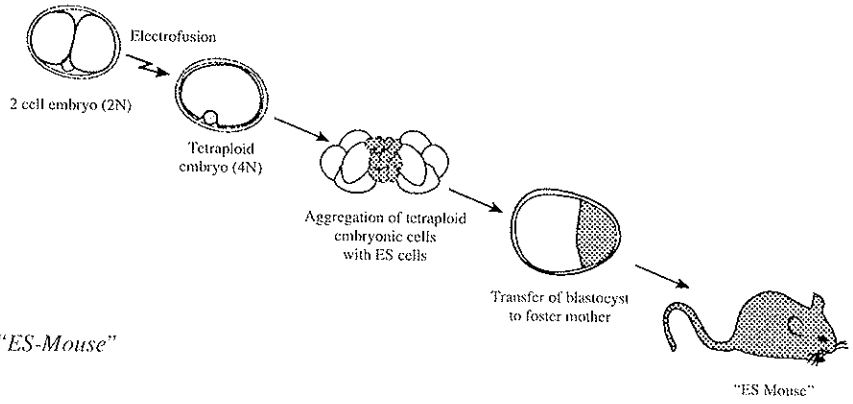

***Fig. 3** The route to the "ES-Mouse"*

III. FUNCTION OF GROWTH FACTOR RECEPTORS

Growth factor receptors play a critical role in the regulation of normal cell proliferation and differentiation and their oncogenic derivatives are causally involved in tumor development. To investigate the function of normal and mutant growth factor receptors during mouse development, we expressed ectopically normal and mutant tyrosine kinase receptors in embryonic stem (ES) cells and during hematopoietic development. This work is partly carried out in collaboration with Axel Ullrich's laboratory in Munich. The normal human epidermal growth factor receptor (HERc), chimeric EGF-R/v-*erb*B (HER*erb*B) or EGF-R/v-*fms* (HER*fms*) receptors and the viral v-*erb*B oncogene were transferred into ES cells and bone marrow derived hematopoietic stem cells. In the chimeric receptors, the high transforming capacity of the oncoproteins is regulated by the extracellular EGF receptor ligand-binding domain. In other experiments aiming to modulate receptor function we have either expressed a dominant-negative EGF-R mutant (HER-CD533) in transgenic mice or are attempting to inactivate the function of the insulin-like growth factor II receptor (IGF-IIR) by homologous recombination in ES cells.

1. Ectopic expression of normal and mutant epidermal growth factor receptors in the mouse hematopoietic system

Thomas von Rüden and Laura Stingl

To analyze the function of normal and mutant EGF-Rs on hematopoietic development *in vivo*, lethally-irradiated mice were transplanted with bone marrow cells (BMC) expressing HERc, HER*erb*B or v-*erb*B. Transplantation of BMC expressing HERc had no effect on normal blood cell development, however, HERc expression could not be maintained in the reconstituted mice despite successful gene transfer. In contrast, reconstitution with BMC expressing the partially ligand-independent chimeric HER*erb*B receptor induced a severe mast cell disease characterized by massive infiltrations of connective tissue mast cells (CTMC) in several visceral organs. An even more severe phenotype was observed in mice expressing the v-*erb*B oncogene. In addition to the mast cell disease which appeared after a short latency, some primary recipients showed symptoms of an acute myelogenous leukemia (AML) similar to that frequently found in patients suffering from malignant mastocytosis. Both hematological disorders are transplantable; secondary recipients of BMC expressing v-*erb*B developed AML regardless of whether the leukemic phenotype was evident in the primary host, whereas HER*erb*B expressing BMC transmitted the mast cell disease.

Transfer of these receptors into W/W^v mast cells lacking functional c-*kit* receptors rescued their defective differentiation into CTMC. This suggests that when normal and mutant EGF-Rs are expressed ectopically on BMC they may utilize signal transduction pathways coupled to the c-*kit* receptor. Ongoing experiments address the question whether ectopic expression of such receptors could also restore the *W* defect at the stem cell level. W/W^v BMC expressing v-*erb*B have been transplanted into W/W^v mice and long-term reconstituted recipients will be analyzed for a potential cure of the *W* associated hematological disorders.

2. Transfer of KIT receptor into the fetal hematopoietic system of W/W^v mice

Warren Alexander and Uta Möhle-Steinlein

In an attempt to rescue the hematopoietic defects of W/W^v mutant mice, we have used infection of mutant fetal liver cells with c-*kit* retroviruses and subsequent transplantation of these cells via placental injection into midgestation embryos. From a large number of mutant mice born, a few exhibited improved blood parameters and also carried vector sequences in peripheral blood cells. Presently we are trying to prove expression of the introduced *kit* gene and are designing new strategies for an efficient way to cure the defects in W-mutant mice.

3. Expression of colony stimulating factor-1 receptor in myeloid and T lymphoid cells

Thomas von Rüden in collaboration with Guy Mouchiroud, Lyon and Kurt Ballmer-Hofer, Basel

The human colony stimulating factor-1 receptor (CSF-1R) was introduced into hematopoietic cell lines of myeloid and T-lymphoid origin, both of which normally do not express the CSF-1R. We could show that CSF-1R expressed ectopically may function on certain myeloid cells suggesting the presence of signal transduction pathways which can be utilized by that foreign receptor. In contrast, it appears that T-lymphoid cells lack such a signalling mechanism. Interestingly, the myeloid cells expressing CSF-1R could be adapted to grow in high concentrations of serum without IL-3 or CSF-1. Recent experiments have shown that insulin-like growth factor-I (IGF-I), but not IGF-II, insulin, EGF or PDGF, can stimulate proliferation of such cells whereas it does not stimulate cells ectopically-expressing the EGF-R. Currently, we are addressing the question of whether such IGF-I binding proteins may utilize the exogenous CSF-1R to transduce mitogenic signals.

4. Ectopic expression of normal and mutant tyrosine kinase receptors in ES cells

Ulrike Burkert and Marion Fung

To investigate the effect of mutant tyrosine kinase receptors on embryogenesis, we have attempted to express ectopically in ES cells the constitutively active v-*erb*B oncogene as well as the inducible chimeric growth factor receptors HER-*erb*B and HER*vfms*. Several heterologous promoters including the inducible metallothionine promoter (MT), a constitutive promoter (PGK-1) and retroviral vectors such as NTK-v-*erb*B were used. All vector constructs conferred efficient expression of the receptor mutants in fibroblasts.

Following the introduction of v-*erb*B into ES cells by retroviral-mediated gene transfer, all clones analyzed expressed the phosphorylated protein. Since expression of v-*erb*B has been shown to drastically affect hematopoietic differentiation (see section III.1), our studies focused on the hematopoietic differentiation capacity of the v-*erb*B-expressing ES cells *in vitro*. In comparison to wild-type cells, the potential of these clones to develop into various myeloid lineages *in vitro* was not altered. Currently, we are testing whether functional v-*erb*B protein is retained during the *in vitro* differentiation process.

Expression of the inducible HER-*erb*B protein was achieved only with the constitutive PGK-1 promoter. Three out of 60 selected ES clones expressed HER*erb*B receptors on the cell surface, suggesting that expression of this chimeric protein may be dependent upon the site of vector integration. Expression of HER*erb*B did not alter ES cell morphology, growth rate or *in vitro* differentiation potential, despite culture in the presence of the ligands EGF or TGF-α. However, subcutaneous injection of ES cells displaying the HER*erb*B protein into syngeneic mice led to an increase in tumor growth rate, indicating that the transforming potential of HER*erb*B may be active in ES-derived teratocarcinomas. To explore the biological effects of HER*erb*B *in vivo*, we are currently attempting to generate mice overexpressing the chimeric receptor.

The PGK-HER*vfms* construct gave rise to high levels of HER*vfms* RNA in ES cells. However, in contrast to HER*erb*B, neither cell surface nor cytoplasmic HER*vfms* protein could be detected. This block in ectopic receptor expression was not overcome by *in vitro* differentiation.

5. Expression of a dominant-negative mutant to block EGF-R function

Uta Möhle-Steinlein in collaboration with
Axel Ullrich, Munich

To interfere with growth factor receptor signalling, a dominant negative mutant of the human EGF-R (HER-CD533) was used, which was first characterized for its inhibitory function in tissue culture. Two transgenic mouse lines were established, one of which showed efficient and inducible expression of the HER-CD533 transgene in several organs. Presently we are assessing the possible biological consequences of ectopic CD533 expression through immunocytochemical and *in situ* RNA analysis as well as by stimulating physiological processes which are dependent on EGF-dependent signalling.

6. Inactivation of the insulin-like growth factor II receptor gene

Marion Fung in collaboration with Denise Barlow

The insulin-like growth factor II receptor IGF-IIR / large mannose 6-phosphate receptor (MPR300) is expressed both during mouse development and in the adult animal. This bifunctional receptor plays a role in regulating IGF-II levels and in lysosomal targeting. However, its exact biological function is unclear since this receptor also binds other growth factors such as latent TGF-β. The *Igf2r/MPR300* gene has been shown to be imprinted in mice and is possibly identical to the *Tme* gene, which causes embryonic lethality at day 15 (see report by Denise Barlow).

To investigate the biological role of IGF-IIR/MPR300, we have inactivated the *Igf2r/MPR300* gene through homologous recombination in ES cells using a nlsLacZ construct. In this targeting vector, LacZ expression is driven by the endogenous *Igf2r/MPR300* promoter. Correct targeting to the *Igf2r/MPR300* locus was achieved at a frequency of 1 in 30 *neo*-resistant clones (Fig. 4). Consecutive inactivation of the second allele was achieved by selection in high G418 concentration. In the next few months, we will attempt to generate *Igf2r/MPR300*-deficient mice and both the expression pattern and the functional effects of the receptor during development will be investigated.

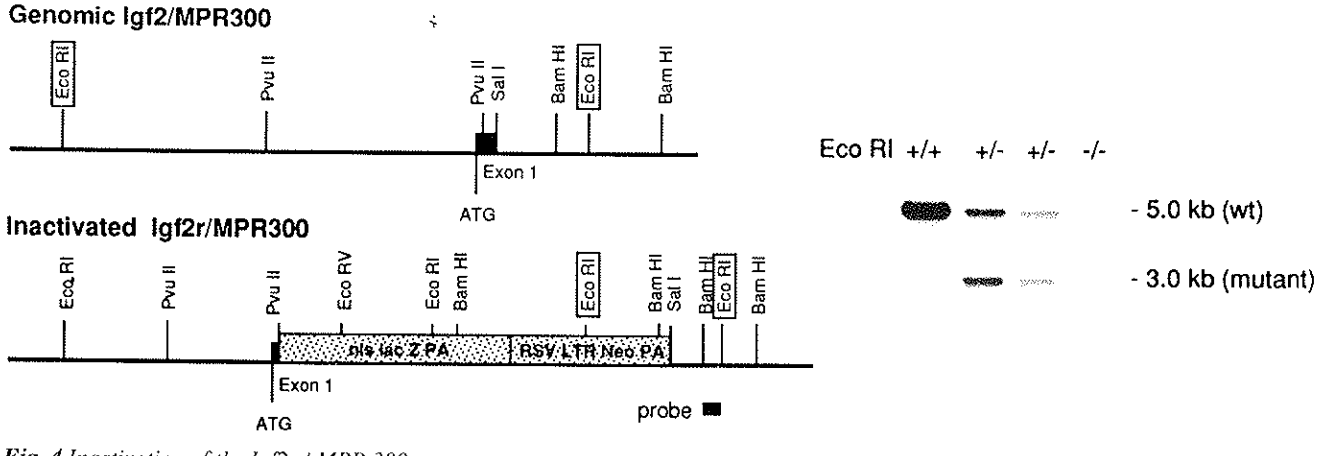

Fig. 4 Inactivation of the Igf2r / MPR 300 gene

IV. COOPERATIVITY BETWEEN HUMAN FOAMY VIRUS AND HUMAN IMMUNODEFICIENCY VIRUS

Adriano Aguzzi and Ingrid Anhauser

Although infection by HIV-1 is the primary cause of AIDS, co-factorial agents may be involved in the pathogenesis of the disease. A potential candidate is Human Foamy Virus (HFV), whose seroprevalence reaches up to 20% in East African patients suffering from AIDS and AIDS-related complexes. Although evidence for a role of HFV in human disease is accumulating, HFV has not yet been shown to be the specific causative agent of a human disease. HFV contains a complex genome similar to that of lentiviruses and includes a transcriptional activator, the *bel*-1 gene. We have recently shown that HFV transgenic mice develop a myopathy and a vacuolar leukoencephalopathy closely resembling that of AIDS patients, thereby proving that HFV can exert pathogenic effects in a mammalian host.

We are presently analyzing a possible cooperation between HFV and HIV-1 in a transgenic mouse system. Double transgenic mice have been generated containing (i) the *bel*-1 gene of HFV under the control of the homologous LTR and (ii) a lacZ reporter gene under the control of the U3 region of the HIV-1 LTR. Expression of the transgenes has been studied by *in situ* RNA hybridization, immunohistochemistry for *bel*-1 and β-galactosidase, and X-gal histochemistry. The results demonstrate that cross-transactivation of the HIV-1 LTR by *bel*-1 takes place *in vivo*. Expression of the reporter gene was detected in neurons, indicating that *bel*-1 can induce a shift in the tissue specificity of HIV-1 expression. Rather than being a general phenomenon, cross-transactivation appears to be restricted to specific neuronal populations. In order to assess the clinical relevance of these phenomena, we will study the prevalence of HFV infection in AIDS patients suffering from neurological diseases using immunochemical techniques and PCR analysis. The outcome of our efforts could have significant clinical implications, if HFV proved to be a co-factor of triggering AIDS in asymptomatic HIV carriers.

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The Group Leaders

REGULATION OF PROTEIN SERINE/THREONINE KINASES DURING EARLY G_1

Lisa M. BALLOU	Group leader
Najwa CHERRADI	Postdoc (until April 1992)
Helga EDELMANN	PhD student (since Dec. 1991)
Claudia PETRITSCH	PhD student

Introduction

S6 kinases are serine/threonine-specific enzymes that phosphorylate the S6 protein of 40S ribosomal subunits. These kinases are inactive in non-growing (G_0) cells but rapidly become activated when cells are stimulated and enter G_1. During this time S6 becomes highly phosphorylated and there is an increase in protein synthesis that is required for cells to progress through S phase. Two distinct families of S6 kinases have been identified. The 70 kDa enzymes (M_r=65-85 kDa) are the major S6 kinases in fibroblasts and have been found in tissues from rat, mouse, human, chicken, etc., as well as in Xenopus laevis eggs. The 92 kDa S6 kinases (M_r=85-92 kDa), also called rsk, were first identified in Xenopus eggs and later found in somatic cells from higher species. Enzymes in the two families are similar in that (1) they are activated in vivo by growth factors, phorbol esters, oncogenes, etc.; (2) activation requires phosphorylation of serine/threonine residues; and (3) phosphatases 1 and 2A inactivate the kinases in vitro. However, they have distinct protein structures and kinetics of activation, and they lie on different signalling pathways. In particular, 92 kDa S6 kinases are activated by MAP kinases, but the 70 kDa enzymes are not. We would like to understand how activation of the 70 kDa S6 kinase contributes to the mitogenic response and are interested in identifying the cellular mechanisms that control the activity of the enzyme.

Identification of the S6 kinase activator

Lisa M. Ballou

It appears that the 70 kDa S6 kinase participates in a phosphorylation cascade with at least one serine/threonine kinase linking receptor tyrosine kinases with S6 kinase. I am interested in identifying the S6 kinase kinase, with the ultimate aim of tracing all the steps in this kinase cascade. To do this, I have used an in vitro assay that should detect kinases that reactivate S6 kinase. Briefly, purified S6 kinase is dephosphorylated and inactivated with a phosphatase. Then the protein is incubated with Mg-ATP plus fractions derived from resting or stimulated cells and reactivation of S6 kinase is assayed. In the past year this reactivation assay was carried out under a multitude of conditions using crude cell homogenates, low-speed supernatants, and cell extracts fractionated and concentrated on Blue Sepharose, Mono Q and Mono S. Reactivation of S6 kinase was not observed under any condition. Recently, four in vivo phosphorylation sites at the C terminus of the enzyme were identified (Ferrari et al., PNAS 89, 7282 (1992)). They are clustered in a putative autoinhibitory domain of the enzyme and are surrounded by consensus sequences recognized by cdc2-like and MAP kinases. Since all known MAP kinases are activated by tyrosine phosphorylation, I tested material eluted from anti-phosphotyrosine antibodies as a potential source of S6 kinase kinase. Material precipitated by suc-1 beads, which bind some cdc2-like kinases, was also tested. No reactivation was observed with these preparations, either alone or in combination. One possible explanation for these results is that the phosphatase-treated S6 kinase used as a substrate in the reactivation assay is in a conformation that cannot be reactivated. In the future I will produce large amounts of the 70 kDa S6 kinase in insect cells and use it as a substrate to screen for enzymes that phosphorylate the protein. Promising kinases will then be tested in a modified reactivation assay.

Interaction of cAMP and S6 kinase activation pathways

Claudia Petritsch

Growth of fibroblasts is associated with decreased levels of cAMP early in G_1. In addition, agents that increase intracellular cAMP levels, such as phosphodiesterase (PDE) inhibitors or activators of adenylate cyclase, inhibit growth of fibroblasts, T lymphocytes, and other cell types. Interestingly, pretreatment of cells with non-specific PDE inhibitors such as theophylline or SQ20,006 also completely inhibits the EGF-induced activation of the 70 kDa S6 kinase. Activation of the 92 kDa S6 kinase and its activator, MAP kinase, is not affected. Thus, the PDE inhibitors do not disrupt all EGF receptor-mediated processes. These effects are similar to those observed with rapamycin, an immunosuppressant. Several other PDE inhibitors and high concentrations of forskolin also inhibit S6 kinase activation, suggesting that the block might be due to increased cAMP levels. In eukaryotic cells, almost all of the effects of cAMP are mediated by cAMP-dependent protein kinase (PKA). Since neither cAMP nor PKA inhibits the 70 kDa S6 kinase in vitro, a simple hypothesis to explain the effect of PDE inhibitors would be that activated PKA phosphorylates an enzyme in the S6 kinase activation pathway to inhibit it. To test this hypothesis, I examined the effect of theophylline on S6 kinase activity in an S49 mouse lymphoma cell line that lacks PKA. PDE inhibitors still block S6 kinase activity in these cells, suggesting that PKA is not the mediator of this process. In the future, specific PDE inhibitors will be tested and the role of cGMP and Ca^{2+}/calmodulin in S6 kinase inhibition will be examined.

Cell cycle regulation of S6 kinase

Helga Edelmann

Much of the work on S6 kinase activation has been carried out on fibroblasts that are arrested in G_0 by serum deprivation or contact inhibition. Addition of mitogens to these cells causes as much as a 50-fold increase in S6 kinase activity during the G_0/G_1 transition. There is evidence that the enzyme might also be active at other points in the cell cycle. For instance, rapamycin, which inhibits the 70 kDa S6 kinase, delays entry of fibroblasts into S phase (Chung et al., Cell 69, 1227 (1992)), suggesting that the enzyme is active in late G_1. In addition, it has been shown that the kinase is active in M phase during meiotic maturation of Xenopus laevis oocytes (Lane et al., EMBO J. 11, 1743 (1992)), suggesting that it might also be active during mitotic M phase. To address these questions, I will examine the cell cycle regulation of S6 kinase. Cells will be arrested either in G_0 by serum starvation or at the G_1/S boundary by mimosine, or a pure population of G_1 cells will be collected by elutriation. After release from the growth block, synchrony of the growing cells will be followed by FACS analysis. Activity of the 70 kDa S6 kinase will be measured in immunoprecipitates using antibodies specific for the enzyme. The phosphorylation pattern of the in vivo labelled enzyme will be examined on 2-dimensional tryptic phosphopeptide maps. mRNA and protein levels will be measured on Northern and Western blots, respectively. Finally, the intracellular localization of the enzyme will be examined by immunofluorescence to see if it changes during the cell cycle. Results from these experiments will provide important information about the role of the 70 kDa S6 kinase during cell cycle progression.

MAMMALIAN DEVELOPMENTAL GENETICS

Denise P. BARLOW	Group Leader
Carmen DeNEWRKLA	Technician
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Reinhard STÖGER	Ph.D student

Introduction

Developmental processes such as growth, differentiation, senescence and cell death, and processes specific to the mammalian embryo such as the controlled invasion of maternal tissue by the fetus, all involve genes that have the potential to cause severe disturbance to adult biological systems when inappropriately expressed. Thus the study of genes which control mammalian developmental - a process whereby cells acquire their normal adult function, has great potential to identify genes involved in abnormal pathology - a process whereby cells escape from their correct controls. The goal of our research is thus to clone genes that regulate events in normal mammalian embryogenesis and to characterize their role in development and disease.

Our approach rests on the isolation of genes which *control* events in development. For this purpose we use existing mouse mutant strains whose phenotype demonstrates a developmental abnormality. The gene responsible for the developmental mutation is cloned by „positional cloning" approach that uses the chromosomal position of the mutant locus, and flanking DNA markers, as a starting point to isolate the gene. At present we concentrate on a number of developmental mutants that lie within the proximal part of mouse chromosome 17 in a region known as the *t complex*. This region of the mouse genome is densely mapped containing more than 70 DNA markers and 40 developmental mutants[1]. In the last decade the *t complex* has been the subject of an intensive molecular characterization which has greatly simplified our understanding of this system, and of the ways in which molecular genetic techniques can be used to isolate the developmental mutants mapped to this region. We have focussed on a 4 Megabasepair region of the *t complex* and three lines of research based on developmental mutants contained within this region are currently under investigation. These are - the function of **genomic imprinting** in development and disease, the molecular basis of **embryonic implantation** and the molecular basis of male-specific **segregation distortion.**

1. The function of genomic imprinting in development and disease

D. P. Barlow, P. Kubicka, and R. Stöger

The expression of a subset of genes in the developing mouse embryo has been shown to be dependent upon their parental origin. These genes maintain parental-specific expression in diploid cells, such that one locus is expressed and the other is silent, even when both parental loci are genetically identical. Parental-origin effects on gene expression arise through a phenomenon known as genomic imprinting, that is thought to involve a gamete-specific epigenetic modification that can subsequently cause hemizygous expression in diploid cells. Many imprinted genes act during mouse development and parental-specific effects likely to involve imprinted genes have also been implicated in human disease[1]. At present, however, it is completely unclear why the phenomenon of imprinting acts to restrict gene expression during development. We propose to solve this enigma by identifying a large number of imprinted genes and then analyzing their role in developmental. We are doing this in two steps; firstly, to clone one imprinted gene and, secondly, to define the imprinting signal that triggers parental-specific expression and use this information to clone more imprinted genes.

We are pursuing the first step through an analysis of a classical mouse mutation called *Tme* (T-associated maternal effect). This locus encodes an essential embryonic gene that is imprinted and only expressed from a maternally inherited chromosome. Our recent work[ii] has shown that the Insulin-like growth factor type 2 receptor gene (Igf2r) is a candidate for the *Tme* mutation because it is imprinted, maternally-expressed and maps to the same chromosomal position. Homologous recombination experiments (in collaboration with Marion Fung and Erwin Wagner at the IMP) are currently being performed to test if the Igf2r gene is the same as *Tme*.

The second step, of identifying the signal that triggers parental-specific expression, is being pursued by molecular characterization of the maternal and paternal Igf2r genomic loci. Most studies of the molecular basis of genomic imprinting are consistent with the idea that these genes are subject to epigenetic modification during gametogenesis, that leads to hemizygous expression in the diploid embryo. Since methylation is currently the best candidate for this type of modification we have investigated if the Igf2 receptor gene contains parental-specific methylated regions that could act as an imprinting signal. Our studies show that this is, indeed, a possibility. We have identified a putative methylation imprinting signal that is carried by

the expressed, maternal locus[2]. The developmental profile of methylation of this sequence fits the criteria we have applied to identify the imprinting signal, in that modification of this region is both inherited from the female gamete and maintained in the diploid embryo. This result, that suggests that methylation is necessary for expression of this gene, is in contrast to other experiments in which methylation is associated with repression. Experiments are in progress to derive transgenic mice containing the modified region to test this methylation model for imprinting the Igf2r gene.

2. The molecular basis of embryonic implantation

N. Schweifer

Implantation of the mammalian embryo is an invasive process that requires a balanced interaction between two tissues - the embryonic trophoblast and the maternal uterus. The genes that regulate the invasive behavior of embryonic tissue have not been identified, but it is predicted that similar genes may also play a role in the behavior of invasive tumors. Thus an understanding of the molecular basis of embryonic implantation may elucidate events in tumor pathology.

Homozygous mouse embryos bearing a mutation at the recessive t^{w73} locus cannot implant and subsequently die 8 - 9 days after fertilization. The trophoblast of this mutant apparently acts normally during attachment to the uterine wall, but is considered to be deficient in invasive ability from cytological studies[iii]. As a first step towards the goal of cloning the t^{w73} locus a YAC (yeast artificial chromosome) contig has been constructed spanning the t^{w32} deletion of mouse chromosome 17. This deletion removes approximately 1 cM of DNA including the t^{w73} gene[iv]. The YAC clones are currently being used to identify novel coding sequences from cDNA libraries that will be tested for expression in ES cell lines established from homozygous t^{w73} embryos.

3. The molecular basis of male-specific segregation distortion

D. P. Barlow

Chromosome 17 exists in two variant forms in wild populations of *Mus musculus*. Eighty per cent of mice contain the „*wild type*" form of this chromosome and 20% contain a form known as the „*t haplotype*." These two types of chromosome 17 differ from each other over the proximal 30 - 40 Mb, because of the presence of four neighboring inversions. The structure of the *t haplotype* chromosome, and other factors, resulted in the gradual accumulation of a large number of mutant genes within the *t complex* region. These genes have been shown genetically to affect development and differentiation in diverse biological systems and some have now been cloned[v].

Male mice heterozygous for the *wild type* and *t haplotype* forms of chromosome 17 show a drastic distortion of the expected Mendelian segregation ratio in backcrosses, but in an unexpected direction. Up to 99% of progeny derived from a (+/t) male mated to a (+/+) female will inherit the paternal *t haplotype* chromosome. At least four interacting loci located within the t complex region control this phenomenon. We have used positional cloning to identify one of these genes located at the *Tcd-3* locus (t complex distorter 3). This gene named Tcte-2 (t complex testes expressed) is only expressed during male meiosis and is not expressed from a *t haplotype* chromosome. Genomic and cDNA cloning has shown that the locus is in excess of 100 kb and expresses multiple, differentially spliced mRNAs of varying sizes. Only one of these mRNAs contains an open reading frame, and its role in meiosis and in segregation distortion is currently being studied.

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PERIODIC TRANSCRIPTION DURING THE YEAST CELL CYCLE

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Introduction

The regulation of the eukaryotic cell cycle appears to involve mainly posttranscriptional events whereby specific kinases are thought to modify targets, which then allow the cell to progress through the cell cycle. An intriguing possibility, however, is that cell cycle-regulated transcriptional activation may be involved in the timing and ordering steps of cell cycle regulation. We are testing this hypothesis using the yeast *Saccharomyces cerevisiae* as a model system by isolating all cell cycle dependently expressed genes and test them for a possible function in cell cycle regulation.

During the first 2 1/2 years at the IMP we screened 60% of an ordered phage library, which contains 80 to 90% of the haploid yeast genome, for genes whose expression was dependent on cell cycle. Using an RNA screen that we developed for this purpose we were able to obtain a collection of transcriptional units which showed cell cycle dependent expression. Some of these genes are transcribed at cell cycle stages at which no other yeast gene has yet been attributed. During the last two years we began detailed studies of some of the genes in our collection. For these studies we selected some of those genes that are expressed either in very early G1 or in mitosis. In very early G1 the cell has just completed mitosis but is not yet committed to the next division cycle. At this stage genes may be transcribed that have a specific role in controlling important events before the cell reaches the START point in G1 when the cell has to decide about its developmental fate. In mitosis a cell has to pass through complex stages like nuclear division, where two equal sets of chromosomes have to be distributed under precise conditions, and cytokinesis, when two cells are produced that have to be provided with equivalent amounts of components sufficient to support life for both cells.

In the last 12 months we concentrated on five genes, ***EGT2***, ***MST1***, ***MST2***, ***HSP30*** and ***KIN3***. ***EGT2*** is a gene that is transcribed very early in G1, where it may fulfil a yet unknown function. ***MST1*** and ***MST2*** and also ***KIN3*** are transcribed in mitosis. While the ***MST*** genes and the heat shock inducible ***HSP30*** encode membrane proteins with a possible receptor function, ***KIN3*** encodes a protein kinase which, according to preliminary data, seems to interfere with ordering processes in mitosis when overexpressed.

Characterisation of Genes Specifically Expressed in Mitosis

Wilfried Rossoll and Tillman Schuster

Most genes that we identified in our screen for cell cycle regulated transcripts in the yeast *Saccharomyces cerevisiae* show strong expression at the transition from G1 to S-phase. Two genes, however, appeared to be specifically expressed in mitosis. This is the first time that in *S. cerevisiae* transcriptional activation could be assigned to this point of the cell cycle. ***MST1*** and ***MST2*** (mitosis specific transcript) encode two highly related proteins with seven putative membrane spanning domains. Both gene products are localised in the plasma membrane. While expression of both ***MST*** genes peaks in anaphase, ***MST1*** but not ***MST2*** is super induced by heat shock. The fact that deletion of both genes has no effect on growth might be caused by the existence of a homologous gene which could rescue the ***mst-*** strains. In an attempt to identify this gene we employed a search for synthetic lethal mutations in yeast cells with no functional ***MST*** genes. We screened more than 200.000 colonies but were not able to find a mutant which depends on expression of an ***MST*** gene. The reason for this outcome may either be redundancy, or these genes are not required for the cell under the conditions used in our screen.

Until recently the most closely related protein sequence found in the database was the light-driven proton pump bacteriorhodopsin (ca. 52% similarity). However, when the entire sequence of chromosome III of *S. cerevisiae* was published (Oliver et.al., 1992), a comparison with the translated sequence revealed an open reading frame with a similarity of 57 to 61% and an identity of 33 to 37% to **Mst2** and **Mst1,** respectively. It also contains seven putative membrane spanning domains. Recently it was reported that this open reading frame encodes a protein of 30 kD that is induced by entry into stationary phase as well as by heat shock (M. Regnacq and H. Boucherie). It is not essential for growth and no similarities to other known proteins have been reported by other laboratories. Probably it is identical to the novel heat shock protein **Hsp30** which has been identified in plasma membrane fractions of heat-shocked and stationary cultures (B. Panaretou and P. W. Piper). Analysis of the sequence alignments shows that the regions, which are probably exposed at the outside of the plasma membrane, are identical in all three genes. If these genes belong to the large class of heptahelical receptors it would suggest that all three proteins bind the same ligand. Initial experiments have shown that ***HSP30*** is not cell cycle regulated but induced by pheromone arrest. Currently, we are investigating whether deletion of ***HSP30*** in an ***mst1-***, ***mst2-*** background has any effects on growth under different conditions.

The third gene that has been identified in *S. cerevisiae* as being expressed exclusively in mitosis is ***KIN3***. ***KIN3*** has been found as an open reading frame adjacent to ***CDC15*** (A. Amon, pers. comm.). It encodes an Ser/Thr protein kinase (Jones and Rosamund, 1990) and turned out to be closely related to ***nimA*** (never in mitosis), a gene of the filamentous fungus *Aspergillus nidulans* which is expressed specifically in mitosis (61% similarity / 43% identity on the protein level). In *A. nidulans* both the mitotic kinase **p34^{cdc2}** and **nimA** are required for progression into mitosis. In contrast to ***nimA KIN3*** is not essential for growth but constitutive overexpression seems to lead to defects in cytokinesis and nuclear division. Currently we are studying the effects of ***KIN3*** overexpression by *in situ* immunofluorescence microscopy with anti-tubulin antibodies.

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Regulation of the MST-Genes

Axel Behrens and Tillman Schuster

The ***MST***-genes belong to the first three genes in *S. cerevisiae* found so far that show mitosis-specific expression. Studying the regulation of these genes may reveal new regulatory components that are necessary for gene expression at this cell cycle stage. It also may lead to the understanding of how the regulation of these genes is dependent on the events that control this cell cycle stage. Therefore, one of the first questions we wanted to answer is whether ***MST***-gene activity is dependent on ***CDC28*** kinase function. To learn when in the cell cycle activation of ***MST*** gene transcription occurs we arrested cells of a yeast strain, which lacks four G2 cyclins and is rescued by a Gal promoter driven ***CLB2*** gene (***clb1-***, ***clb2::Gal1-CLB2***, ***clb3-***, ***clb4-***), at the G2/M phase boundry. At this point no ***MST*** transcripts are detectable. Only shortly after release from the arrest by adding galactose the ***MST*** genes are activated. This suggests that activation of ***MST*** transcription is dependent on mitotic kinase function.

To test when in the cell cycle, and by which cell cycle function ***MST*** transcription is turned off, we used a yeast strain lacking three G1 cyclins (***cln1-***, ***cln2-***, ***cln3-***) which arrests at START in G1. This strain contains a GAL promoter driven ***CLN3*** gene which releases the cells from the arrest after addition of galactose to the media. Using temporal staged RNA blots we observed that both ***MST1*** and ***MST2*** seem to remain derepressed and their RNAs appear to accumulate during the arrest. This is in contrast to the results we obtained using the pheromone arrest. Here, both genes were completely repressed. Thus, the function of the CDC28/G1-cyclin-complex seems to be involved in controlling the cell cycle dependent repression of the ***MST***-genes.

In addition we want to identify the factors that mediate the cell cycle specific controls of the ***MST*** genes. Therefore, we decided to examine their promotors for possible regulatory elements. Since we found that ***MST1*** in contrast to ***MST2*** is not only cell cycle regulated, but also super induced by heat shock, ***MST2*** was chosen to be used in our studies. ***MST2*** promoter sequences carrying different deletions are cloned in front of a reporter gene and introduced into the genome of a wild type strain. The deletions will then identify the sequences that are necessary for induction and/or repression of the reporter gene. These sequences can be used to search for specific DNA binding factors.

EGT2 is Expressed in a Narrow Window Between Mitosis and START.

Branislav Kovacech and Tillman Schuster

EGT1 and ***EGT2*** are genes with yet unknown function that are transcribed in early G1 (early G1 transcripts) at a cell cycle stage, at which no other yeast gene has yet been attributed. Both genes are tightly cell cycle regulated which may indicate that their regulation is of importance for their correct function at this cell cycle stage. Since our work is directed to the identification of genes that are trancriptionally regulated in a cell cycle dependent fashion and also involved in the regulation of the cell cycle, ***EGT1*** and ***EGT2*** became candidate genes to study. This work is focussed on the characterisation of the ***EGT2*** gene. We are studying the regulation of the gene and trying to find what function it may have for cell growth.

The G1 specific expression of ***EGT2*** was studied by hybridising temporal staged RNA blots with radioactively labelled ***EGT2*** probes. Hereby, three different methods were used to synchronise cell populations. In the first two cases we used the release from pheromone arrest in G1 and the release of a cdc15-mutant that arrests in mitosis if kept at the non-permissive temperature. In the third case we used cells harbouring the genotype ***cln1-***, ***cln2-***, ***cln3-*** which are rescued by a functional ***CLN1*** gene driven by a GAL1 promoter. They were arrested by culturing in raffinose containing medium (in the absence of galactose). The culture was synchronously released from the G1 arrest by adding galactose. These three experiments allowed us to conclude that the ***EGT2*** gene is transcribed in a very narrow window in the cell cycle. Its expression begins very late in mitosis (or immediately after mitosis) and is already repressed before START at the pheromone arrest point in the G1-stage of the cell cycle when ***EGT1*** is still active.

EGT2 encodes an unknown protein with a length of 1003 amino acids. It contains eight 35 amino acid repeats of a novel type. Searches in the available databases did not reveal any homologies to known motifs nor any hints for a function.

Since ***EGT2*** is expressed exclusively in very early G1, we checked the possibility whether the gene product participates in some processes that take place in the G1-stage. We tested if the disruption of the ***EGT2*** gene has an influence on budding site selection and on α-factor response. But no changes in cell morphology or growth were detected. FACS analysis of ***EGT2+*** and ***egt2-*** cells, however, revealed that ***egt2-*** cells tend to form small clusters of four members which do not separate after sonication. This implies that the ***EGT2*** gene product could participate in processes that separate the cells after nuclear division. This putative function is under further investigation.

Since the localisation of the gene product can provide a hint for a function, we are going to localise the ***EGT2*** gene product in the cell using *in situ* immunofluorescence microscopy. One possibility why we have not yet revealed the function of the ***EGT2*** gene is functional redundancy. To bypass this problem we are going to search for synthetic lethal mutants in cells harbouring an ***egt2-*** background. This approach will allow us to identify mutants that depend on an ectopically expressed ***EGT2*** gene.

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BIOLOGICAL CHEMISTRY

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„Transferrinfection: Receptor-mediated gene transfer"

Karl Mechtler and Ernst Wagner (in collaboration with the groups of Max Birnstiel and Matt Cotten, IMP, and David Curiel, Chapel Hill)

We have developed a novel gene transfer system which uses the receptor-mediated endocytosis route to import DNA into mammalian cells (ref.1-3). Essential steps of the gene transfer include (i) efficient binding to the cell and uptake into endosomes, and (ii) release to the cytoplasm and to the nucleus. DNA gene constructs complexed with polylysine-conjugated transferrin (which serves as a ligand for the transferrin receptor) and polylysine-conjugated, replication-defective adenovirus dl312 (which serves as an endosome-disruption agent) have been delivered to and expressed at very high level in a large proportion of target cells (80% to almost 100% in a variety of cell lines, 30% to 80% in primary endothelial cells, primary fibroblasts and primary melanoma cells). Gene transfer *in vivo* has been demonstrated by local injection of the complexes into the liver of rats, which resulted in a luciferase reporter gene expression of 4×10^6 light units/injection site. Application of the complexes via the bile duct system resulted in a similarly efficient gene transfer to a more extended area of the liver. In a model for *in vivo* gene transfer to endothelial cells of blood vessels, gene expression in tail veins of mice was observed after local application with the blood supply clamped off for 20 min.

In contrast to conventional viral vectors the delivered gene is not part of the viral genome but carried on the exterior of the adenovirus, being therefore far less restricted to a particular size or sequence of the DNA to be delivered. The use of replication-defective and chemically inactivated adenovirus avoids most of the potential hazards associated with recombinant viral vectors. The gene transfer technique developed in our lab may become a powerful tool for gene therapy, particularly in cases where large genes have to be transferred and *ex vivo* gene transfer is possible. The system seems to be most applicable for cytokine gene transfer into tumors in a „Cancer vaccine" approach because it has several advantages over established protocols, in that i) a high percentage of primary cells can be targeted, ii) leading to high-level gene expression for at least a week iii) without the need for selecting transfected clones (in collaboration with Georg Stingl, Vienna).

The technique may become even more attractive if a synthetic virus-free gene transfer system can be generated that combines maximum efficiency with optimum safety.

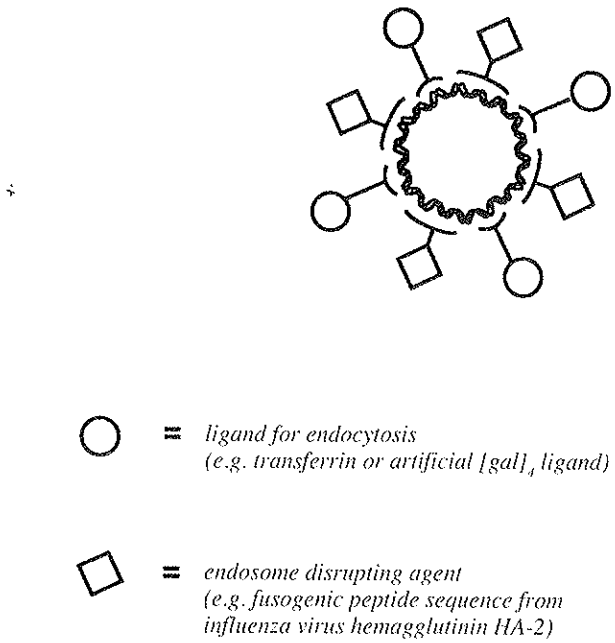

Figure 1. *DNA combination complexes containing receptor ligands and membrane-active peptides.*

„Augmentation of receptor-mediated gene transfer by membrane-disruptive peptides"

Christian Plank, Berndt Oberhauser, Christian Koch, Karl Mechtler and Ernst Wagner

One limiting step to gene transfer by receptor-mediated endocytosis is the exit of DNA from endosomes before being degraded in lysosomes. Viruses escape intracellular degradation by mechanisms induced at the acidic pH of endosomes which lead either to disruption of the endosomal membrane (capsid viruses) or its fusion with the viral membrane (enveloped viruses).

We try to exploit this mechanism for receptor-mediated gene transfer by linking synthetic membrane-disruptive peptides derived from the N-terminal sequence of influenza virus hemagglutinin HA-2 to complexes containing DNA, transferrin and poly(lysine); see **Fig.1**. Binding to the complex is accomplished either by covalent conjugation of the peptides to poly(lysine) or by ionic interaction of acidic versions of peptides with DNA-condensing poly(lysine). The peptides which in the natural context cause fusion with the endosomal membrane, in the context of our artificial complexes disrupt membranes triggered by the change to lower pH and substantially (100 to 500-fold) augment the gene transfer to cell lines of the erythroid lineage (K562 cells) or epithelial lineage (HeLa cells and BNL.CL2 hepatocytes) as well as NIH 3T3 fibroblasts (ref. 4-5). Parameters like pH-specificity and length of the peptides seem to have important influence on their efficiency in membrane-disruption. A dimer of a rationally designed acidic version of the HA-2 peptide was found particularly active in augmenting receptor-mediated gene transfer.

The synthetic virus-like gene-transfer vehicles lack the ordered architecture of viral surfaces thought to mediate optimal exit from endosomes and have 10 to 100-fold lower efficiency than adenovirus-linked DNA complexes. However, the synthetic complexes have been successfully used to generate stably transformed cell lines, which has not yet been possible by using adenovirus-containing complexes.

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THE GENETIC PRINCIPLES OF TUMOR SUPPRESSION

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Introduction

Chromosomal rearrangements in tumor cells are very often the cytogenetic correlates of DNA sequence deletions. As such these deletions are obviously relevant to malignant transformation since they affect regions that are specific for a given cancer type. The genetic information deleted may be required to maintain the non-transformed or non-malignant phenotype. Therefore, such genes are referred to as tumor suppressor genes.

As for the specific locations of such deletions, several forms of human cancer display significant rearrangements in the short arm of chromosome 1. We aim to identify and isolate putative tumor preventing genes from this region. For this purpose we have selected two tumor types, neuroblastoma and primary hepatoma, which display 1p allelic deletions at very high frequencies.

We have chosen a stepwise strategy, consisting of two major parts: i) a detailed genomic DNA analysis, and ii) the detection, cloning, and characterization of genetic elements. The genomic analysis involves the detection of deletions by cytogenetics and loss of heterozygosity analysis, the definition of a region that is consistently lost in tumors, and - most importantly - the generation of a physical map of the region of interest. We designate a detailed physical map a valuable prerequisite for the cloning of genes, since it allows the selection of markers close to the locus of interest.

Important genes are identified and cloned by positional cloning strategies, including the identification and cloning of CpG islands. To date two out of five CpG islands cloned could be mapped to the neuroblastoma consensus deletion. cDNAs have been cloned and sequenced for both of the corresponding transcripts. A subsequent candidate gene approach consisting of structural and expression analyses addresses the question of whether a particular gene may indeed represent a tumor preventing gene.

We have also considered alternative approaches leading to the identification of genes or even directly to a candidate gene. One of these is the mapping of previously unassigned genes which are likely to be involved in genetic defects. Another approach is the analysis of a transgenic mouse line consistently inheriting a neuroblastoma phenotype. This mouse model may possibly reveal important genetic similarities to human neuroblastoma.

Chromosome 1p35-p32 Consensus Deletion in Primary Liver Tumors

Elke Kleiner and Andreas Weith, in collaboration with J. Funovics, Franz-Joseph Hospital, Vienna

A high incidence of chromosome 1p allelic deletions had been found in previous loss of heterozygosity analyses of liver tumors (Simon et al., 1991). We have extended the analyses to a total of 22 hepatomas. Of these, 18 displayed allelic loss in the distal 1p region, underlining the significance of 1p deletions in this cancer type. Moreover, a previously defined consensus deletion could be redetermined and refined owing to the newly identified allelic deletions. The region commonly lost in the 18 tumors is confined to the 1p35-p32 interval (Fig 1). The borders of this consensus deletion are defined by the D1S7 (1p35) and the GLUT1 (1p32) locus, respectively.

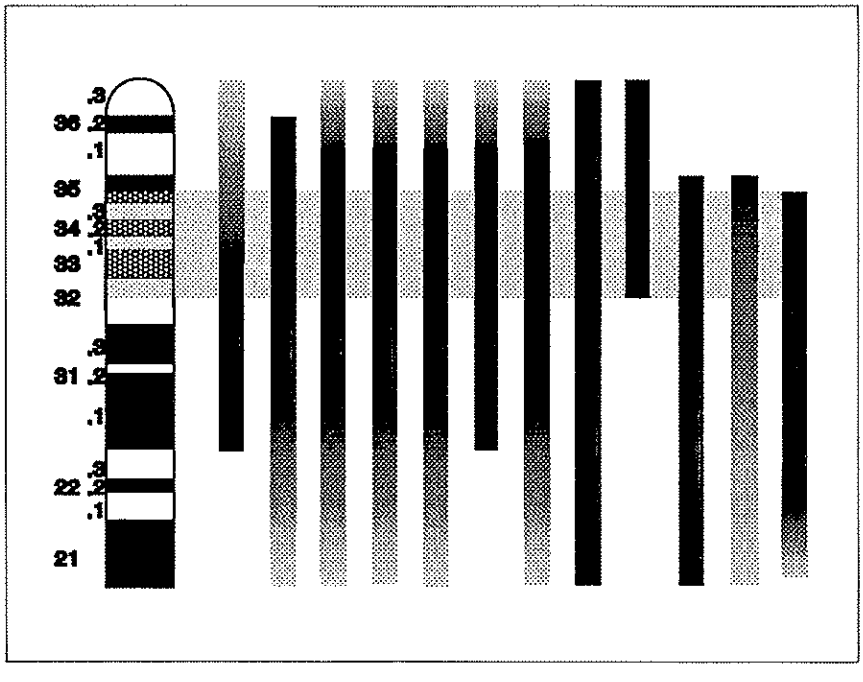

Figure 1. *Chromosome 1p-specific allelic deletions in primary hepatomas. Regions with loss of heterozygosity in twelve different tumors are drawn as black vertical bars. The smallest region of overlap of the deletions is indicated by the shaded horizontal area located between bands 1p35 and 1p32.*

According to the CEPH genetic linkage map (Dracopoli et al., 1991, NIH/CEPH Coll. Mapping group, 1992) the interval between D1S7 and GLUT1 corresponding to the hepatoma consensus deletion spans 19.7 cM. Provided that 1 Mbp corresponds approximately to 1cM, this consensus deletion comprises about 20 Mbp of DNA.

The results of the extended analyses on allelic loss in primary hepatomas clearly demonstrate that the consistently deleted regions in neuroblastomas (see below) and hepatomas do not overlap. We therefore suggest that different genetic elements of the distal 1p arm are involved in the tumorigenesis of the respective malignancies.

A Physical Map for Human Chromosome 1pter-p35

Paula Stapleton, Christoph Barnas and Andreas Weith

Using a series of polymorphic microclone probes (Martinsson et al., 1989, Weith et al., 1992), we had previously been able to determine a consensus deletion in neuroblastomas at 1p36.2-p36.1, that spans approx. 5-8 Mbp of DNA (Weith et al., 1989). Our subsequent attempts to generate a physical map using PFGE resulted in the determination of 13 individual linkage clusters in the entire 1p36 region. These linkage clusters represent sets of 2 to 7 probes that display comparable hybridisation patterns on PFGE blots and therefore are neighboring loci. The 13 clusters contain 63 markers to date and amount to approx. 20 Mbp in size. This may represent more than 70% of the 1p36 band which is roughly calculated being 25 Mbp in length.

The relative order of the linkage clusters remain unknown with some exceptions, the most important ones being three linkage clusters within the consensus deletion since they contain RFLP probes that reside in this region. To create a more detailed physical linkage map of 1pter-35 we are ordering probes representing each of the 13 clusters, as well as probes from other genes of interest in this region. The probe order is being determined using fluorescence in situ hybridisation (FISH) to both metaphase chromosomes and G1 interphase nuclei. In addition, FISH to G1 interphase nuclei will be used to establish the distances between the probes in this region.

A preliminary physical map, using FISH to metaphase chromosomes, has established the order of 5 probes relative to each other and has tentatively assigned positions to 3 others. When this physical map is complete, it will be used to analyse tumour derived cell lines that show alterations in this region. Using this approach we aim to find genes that may be associated with the diseases mapping to this area.

Heir-1: A New Member of the Helix-Loop-Helix Gene Family

Wilfried Ellmeier, Elke Kleiner and Andreas Weith, in collaboration with Adriano Aguzzi

Helix-loop-helix (HLH) proteins are thought to be involved in different developmental processes (reviewed by Jones, 1990). They represent key genes of mammalian muscle differentiation and of the development of sensory organs in the peripheral nervous system of Drosophila. HLH proteins generally function as dimeric complexes, facilitated by their dimerizing HLH motif. Whilst those proteins that also contain a basic DNA binding domain (bHLH proteins) act as transcriptional activators, those without this domain (HLH proteins) counteract the activating function of bHLH proteins. HLH proteins form heterodimers with bHLH, rendering the resulting complex non-functional due to the missing DNA binding domain of the HLH protein.

Heir-1, which was isolated by molecular cloning of CpG islands located in 1pter-p36.1, belongs to the growing family of „Id-like" HLH proteins lacking a basic domain. By Loss of heterozygosity analysis and by FISH it was shown that heir-1 maps to the neuroblastoma consensus deletion. Examination of the expression pattern in different human tissues revealed a high abundance in adult lung, kidney and adrenal medulla, but not in adult brain. Despite its prominent expression in adrenal medulla, the primary target tissue for neuroblastoma, the abundance of heir-1 mRNA in 10 out of 12 analyzed neuroblastoma-derived tumor cell lines is drastically reduced or even absent. Low heir-1 expression was generally found in tumor cell lines with N-*myc* overexpression, whereas the two cell lines displaying high heir-1 levels did not overexpress N-*myc*. Extending the expression studies to the developing mouse embryo, mutually exclusive expression of both genes was also found by in situ hybridization, particularly in the forebrain neuroectoderm. From these results we suggest an inverse correlation between heir-1 and N-*myc* expression in neuroblastoma tumors and in embryonic development.

Ongoing and future experiments will particularly concentrate on 1) transfection of the heir-1 gene into neuroblastoma-derived cell lines which do not express heir-1 but overexpress N-*myc* and on 2) studying the interaction of heir-1 with cellular bHLH proteins. With these experiments we should be able to decipher a potential molecular mechanism for the mutually exclusive expression and to define a role for heir-1 in development and neuroblastoma tumorigenesis.

Chromosomal Localisation of the Human Pax Genes

Paula Stapleton and Andreas Weith, in collaboration with Pavel Urbanek, Zbynek Kozmik and Meinrad Busslinger

Pax genes are a multigene family that share a conserved motif - the paired box - with developmental control and tissue specific genes of *Drosophila*. To date eight murine members of this family have been described (Pax-1 to Pax-8) and their chromosomal locations determined (Walther et al., 1991). Three members (Pax-1, Pax-3, Pax-6) and possibly a fourth (Pax-8) have been associated with previously described murine developmental mutants (reviewed in Gruss and Walther, 1992). Furthermore the human Pax-3 and Pax-6 homologues were found to map to regions of the human genome previously associated with disease loci. This and other evidence lead to the association of Pax-3 with Waardenburg Syndrome Type 1 (Tassabejhi et al., 1992, Baldwin et al., 1992) and Pax-6 with Aniridia (Ton et al., 1991). The involvement of these two Pax genes in human disease illustrates the importance of defining the chromosomal location of the other human Pax homologues to determine if they also show association with any previously described disease loci.

To approach this we decided to use fluorescence in situ hybridisation (FISH) in conjunction with Southern hybridisation analysis of a panel of human/rodent somatic cell hybrids containing known and distinct sets of human chromosomes. This allowed us to localise five of the known human Pax genes and a newly identified one, Pax-9, to their respective chromosomal locations. To complete this work,

the chromosomal band location is being established by mapping the Pax genes in reference to probes that have known chromosomal band positions. We have determined the chromosomal band positions for four Pax genes with reference to two adjacent loci and for two with reference to one probe (Table 1). In the context of the chromosome 1 analysis, we could map Pax-7 to the neuroblastoma consensus deletion. Further functional analyses will be performed to detect an involvement of this gene in any of the diseases with chromosome 1p36-specific rearrangements.

Table 1. Chromosomal Localisation of Pax Genes

Pax Gene	Human Location	Murine Location[1]
Pax-1	20p11	2
Pax-2	10q25	19
Pax-5	9p13	4
Pax-7	1p36.2-p36.1	4
Pax-8	2q12-14	2
Pax-9	14q12-13	n.d.

[1] see Walther et al., 1991, **n.d.** not determined

Neuroblastoma in the Mouse

Christoph Barnas and Andreas Weith, in collaboration with Erwin Wagner and Adriano Aguzzi

In spite of several transgenic approaches towards both the function of the N-*myc* gene and neuroblastoma (reviewed by Aguzzi et al., 1992), a true neuroblastoma mouse phenotype has arisen merely by serendipity in the laboratory of Erwin Wagner. Several mice transgenic for the polyoma middle T oncogene (PyMT) were generated. Whilst two out of four founder animals obtained developed hemangiomas and one did not display any phenotype at all, the fourth developed typical neuroblastoma tumors and passed on this phenotype to its offspring (Aguzzi et al., 1990).

Initial Southern and in situ hybridization analyses had indicated that the genome of the neuroblastoma mice contains a single PyMT integration site though the transgene might have integrated in tandem copies at this locus. From this observation two models for the constant development of the neuroblastoma phenotype may be deduced. Firstly, PyMT may reveal its oncogenic potential specifically in the target tissues for neuroblastoma owing to the coupling to a neuroectoderm-specific controlling element. This assumption may be supported by the observation that PyMT is expressed only in brain and tumor tissue of the mice. Secondly, the phenotype may not be due to the expression of PyMT itself but rather to the functional inactivation of a neuroectoderm-specific gene at the integration locus of the transgene.

We have assessed this question by molecularly cloning the PyMT integration site and characterizing the genomic region in detail. Recombinant DNA libraries were generated from the transgenome in both Cosmid and lambda vectors. To date we have isolated six PyMT-positive clones. All clones contain genomic mouse DNA in addition to transgene sequences. Restriction digest analyses displayed a similar fragment pattern for all six clones suggesting that they were derived from the same locus. When the genomic DNA fragments of the clones were used as hybridization probes, single copy fragments were detected in genomic DNA of various normal mouse strains.

Further experiments will aim to identify non-transgenic coding or controlling elements at this locus and to determine their expression pattern and a possible role in the development of the neuroblastoma phenotype.

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NUCLEAR ONCOPROTEINS AND TRANSCRIPTION FACTORS IN HEMATOPOIETIC CELL DIFFERENTIATION

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Introduction

Hematopoietic cell differentiation involves the highly ordered and controlled proliferation of immature progenitor cells and their commitment and differentiation into fully mature cells of various lineages. Avian retroviruses provided particularly useful tools to investigate various aspects of hematopoietic cell differentiation, since they contain oncogenes which selectively transform cells of distinct hematopoietic lineages. In addition, as a unique advantage offered by the chicken system, hematopoietic cell differentiation and leukemogenesis can be studied *in vitro*, using oncogene transformed, non-established primary cell strains that retained their capacity to undergo apparently normal differentiation in culture.

The focus of research in this laboratory is to study transcription factors which upon oncogenic activation cause leukemia or which play key roles in hematopoietic cell differentiation. However, rather then studying such molecules biochemically and in isolation, we are interested to explore their function within the complex network that regulates cell growth and differentiation. Our major focus to date has been to use chicken bone marrow cells transformed by conditional oncogenes (whose activity can be „switched on and off") as the experimental system where both self-renewal and differentiation can be independently modulated. This basic principle can be applied not only to study the molecular mechanism of action of various oncogenes and to identify the type of hematopoietic cell they transform (see below for v-*erb*A and v-*rel*), but also as a differentiation system to investigate normal pathways of hematopoietic cell development (GATA factors, see below).

1. v-erbA and c-erbA activity in erythroid cell differentiation

In the past several years, we employed the conditional-oncogene approach to study the v-*erb*A oncogene and its molecular mechanism as a dominant repressor of red cell differentiation. Since it became increasingly clear that *erb*A function involved multiple protein-protein interactions, we have now investigated the interplay of *erb*A with other protein factors such as *fos/jun*/AP-1 and RXR. Such interactions appear to be important for v-*erb*A activation as an oncoprotein. Furthermore, our current efforts are directed towards the identification of *erb*A-regulated genes which mediate signals important for the *erb*A-induced phenotype.

erbA interaction with fos/jun/AP-1

Petr Bartunek, in collaboration with M. Karin, University of Southern California, San Diego, USA

We demonstrated before that a retrovirus-transduced and overexpressed c-*erb*A/T3 receptor regulates red cell differentiation in a hormone-dependent fashion: differentiation is blocked in the absence, and induced in the presence, of T3 (Zenke et al., 1990). Furthermore, during T3-induced differentiation, c-*erb*A inhibits erythroblast proliferation (Disela et al.,1991), indicating that a hormone-activated c-*erb*A/T3 receptor also affects the growth properties of such cells. This observation prompted us to investigate a possible interaction of the erbA proteins with components of the *fos/jun*/AP-1 complex that are known to be involved in growth control (see Angel and Karin, 1991, for a review).

It was found that a hormone-activated c-*erb*A/T3 receptor (while efficiently activating T3-dependent promoters) represses *fos/jun*/AP-1-dependent transcription. In the v-*erb*A oncoprotein, however, both activities are lost.

By using a series of v-/c-*erb*A chimeras and specific point mutations, we have now mapped the domain responsible for trans-repression of *fos/jun*/AP-1 by c-*erb*A to the very C-terminus of the *erb*A protein and therefore to sequences that are deleted in v-*erb*A. This domain exhibits properties of a potential amphipatic α-helix, a structure which has been implicated in hormone-dependent transcriptional activation and which is highly conserved among all members of the thyroid/steroid hormone receptor super family (Zenke et al., 1990; Danielian et al., 1992). In the c-*erb*A/T3 receptor these sequences are also engaged in T3 binding (Saatcioglu et al., 1993).

From these studies we conclude that in the c-*erb*A/T3 receptor the same protein domain participates in both transcriptional

activation by hormone and repression of *fos/jun*/AP-1 activity. The loss of this domain by deletion in v-*erb*A leads to the loss of both functions. It appears therefore that the v-*erb*A-specific C-terminal deletion contributes to v-*erb*A oncoprotein activity by two means (1) the loss of transcriptional activator function converts v-*erb*A (when overexpressed) into a dominant repressor of T3-dependent promoters and red cell differentiation (Zenke et al., 1990), and (2) the loss of repressor activity for *fos/jun*/AP-1 (Saatcioglu et al., 1993) ensures that the highly expressed v-*erb*A protein is not deleterious to growth in leukemic cells.

erbA interaction with RXR

Petr Bartunek, in collaboration with Hartmut Beug, IMP, Vienna, H. Stunnenberg, EMBL, Heidelberg, and B. Vennström, Karolinska Institute, Stockholm

As demonstrated before by many groups, the three related receptors for T3, vitamin D3 and retinoic acid (TR, VDR and RAR, respectively) require the auxiliary protein factor RXR for sequence-specific binding to their response elements (for a review see Laudet and Stehelin, 1992). RXR itself represents a receptor for the retinoic acid (RA) isoform 9-cis RA, and is a member of a growing receptor family among which RXRα, RXRβ and RXRγ were isolated. The binding sites of the heterodimeric TR/RXR, VDR/RXR and RAR/RXR complexes are organized as direct repeats of the sequence AGGTCA where the spacing between the repeats is an important determinant of specificity for receptor binding (see review of Laudet and Stehelin, 1992).

From these studies the interesting question emerges as to whether the v-*erb*A oncogene product also binds to RXR to form a heterodimeric v-*erb*A/RXR complex and whether such an interaction is required for its activity as a dominant negative oncogene.

In vitro binding experiments employing vaccinia virus expressed proteins demonstrated that RXR binding to v-*erb*A is impaired due to a specific point mutation in the C-terminal part of v-*erb*A (Barettino et al., 1993). In keeping with this observation, v-*erb*A does not interfere with RAR/RXR-mediated differentiation of transformed erythroblasts (Schroeder et al., 1992, and our unpublished data), whereas the hypertransforming v-*erb*A variant r12v-*erb*A (Damm et al., 1987) efficiently blocks this process. Interestingly, r12v-*erb*A contains the amino acid residue important for *erb*A-RXR interaction reverted to the c-*erb*A-specific sequence which results in a more avid r12v-*erb*A/RXR interaction (Barettino et al., 1993). It appears therefore that the more potent transforming potential of r12v-*erb*A relates to its ability to titrate out RXR. These studies also suggest that the dominant negative activity of v-*erb*A must involve additional properties than simply antagonizing c-*erb*A/T3 receptor function.

Searching for erbA-regulated genes

Karoline Briegel and Gabi Stengl

We have thus far identified 3 red cell-specific genes whose expression is regulated by the v-erbA oncoprotein (Zenke et al., 1988, 1990; Disela et al., 1991). They encode the erythrocyte-specific carbonic anhydrase II (CAII), the anion transporter band3 and the aminolevulinic acid synthase (ALA-S) and are the only v-*erb*A-regulated genes known so far. In addition, experiments performed by Fuerstenberg et al. (1992) indicated that suppression of two of these genes (CAII and band3) accounts for the specific growth requirements observed for v-*erb*A-expressing cells and therefore accounts for only part of the v-*erb*A-specific phenotype. These studies strongly suggest that the v-*erb*A-induced differentiation arrest must involve an additional group of *erb*A-specific target genes which should play a more determining role in erythroid differentiation, for example as regulators of a cascade of erythroid-specific genes.

Potential candidates of such genes were the GATA-1,-2,-3 transcription factors which are thought to be major transcriptional regulators of red cell differentiation (see below). However, none of them was found to be directly regulated by *erb*A.

We therefore searched for *erb*A-regulated genes by differential cDNA cloning. To date several *erb*A-regulated genes were identified, some of which are differentially regulated by v-*erb*A but not by the c-*erb*A/T3 receptor and *vice versa*. A more detailed analysis of these genes is in progress.

2. To approach the function of GATA transcription factors in erythroid cell differentiation.

Karoline Briegel, in collaboration with K.-C. Lim and D. Engel, Northwestern University, Evanston/Chicago

The GATA factors are a family of Zn-finger transcriptional activating proteins in eukaryotes which bind to the consensus DNA sequence WGATAR and are thought to represent major regulators of red cell differentiation (Orkin et al., 1990). Each member (GATA-1,-2,-3) of this family exhibits a distinct pattern of tissue-restricted and temporal expression (Yamamoto et al., 1990; Briegel et al., 1993 and references therein). GATA-1 is predominantly expressed in cells of the erythroid lineage and also in two related cell-types (megakaryocytes and mast cells), whereas GATA-2 is transiently expressed at high levels in immature erythroid cells and in a broad array of both hematopoietic and non-hematopoietic lineages. GATA-3 expression is confined principally to definitive erythroid cells, T-cells and in specific subsets of neurons in the vertebrate central nervous system.

One enigmatic aspect of GATA expression is that during erythroid cell development all three GATA-1,-2,-3 factors (which ostensibly share the same DNA binding site specificity) are simultaneously expressed within the same cell (Yamamoto et al., 1990; Briegel et al., 1993 and references therein). However, the observed, changing ratios of these three factors with respect to one another might suggest that perhaps the fine balance of these factors plays a role in determining decisions during the maturation pathway in erythroid progenitor cells i.e. that expression of one GATA factor at greater or lesser abundance might lead to an increased propensity towards self-renewal *versus* differentiation in the erythroid differentiation program as well as determine the speed of maturation (i.e. accumulation of erythrocyte proteins). In order to empirically address this question, we have ectopically expressed GATA factors (and conditional estrogen-inducible GATA/ER fusion proteins) in normal TGFα-dependent and in ts oncogene-transformed chicken erythroid progenitor cells where both self-renewal and terminal differentiation can be modulated.

Using transient transfection assays, these GATA/ER chimeric factors were shown to possess hormone-inducible *trans*-activation activities. When stably introduced into primary erythroblasts or conditionally transformed erythroid progenitors, GATA-2/ER promoted proliferation and inhibited terminal differentiation in an estrogen-dependent manner (Briegel et al., 1993). These phenotypic effects are specifically due to the action of ectopically expressed GATA-2/ER since erythroblasts expressing GATA-2 were constitutively arrested in differentiation and erythroid progenitors expressing either Gal/ER or GATA-3/ER did not display a hormone-responsive block in differentiation. Thus the GATA-2 transcription factor appears to play a role in regulating the self-renewal capacity of early erythroid progenitor cells.

3. Transformation of chicken bone marrow cells by a conditional v-rel

Guido Boehmelt and Georg Mellitzer, in collaboration with Hartmut Beug, IMP, Vienna and Paula Enrietto, State University of New York (SUNY) at Stony Brook, USA

v-*rel*, the oncogenic version of c-*rel* (originally transduced by the turkey virus REV-T), is a member of the NF-κB/*rel*/dorsal transcription factor family and induces multiple lymphomas in virus-infected birds. To approach v-*rel* oncogene function in hematopoietic cell transformation, a conditional v-*rel* was generated by fusion with the estrogen receptor hormone binding domain (v-*rel*ER) which renders the v-*rel* oncoprotein hormone-inducible, a paradigm which has been successfully applied before for various proteins (Boehmelt et al., 1992, and references therein).

The v-*rel*ER fusion protein functions as predicted, and causes estrogen-dependent (but otherwise unaltered) v-*rel*-specific transformation of avian fibroblasts and bone marrow cells *in vitro*. In a first series of experiments we have now studied several functional properties of the v-*rel*ER protein (subcellular localisation, association with cellular proteins, sequence-specific DNA binding, transcriptional activity) to investigate the molecular mechanism by which v-*rel*ER activity is modulated by hormone (Boehmelt et al., 1992). It was found that the v-*rel*ER protein binds to NF-κB sites in an estrogen-dependent manner, which identified sequence-specific DNA binding of v-*rel*ER as one step critical for activation of its transforming capacity. In addition, a hormone-activated v-*rel*ER rapidly elevated transcription of two cellular genes that are constitutively activated in v-*rel* transformed cells. This finding is also in keeping with v-*rel*ER function as a hormone-dependent transcriptional activator in transient transfection assays. Since v-*rel*ER activity on gene expression parallels the v-relER-specific effects in transformation, we propose that v-*rel*ER (and possibly also v-*rel*) exerts its oncogenic activity at least in part as a transcriptional activator. This contrasts previous studies where, based on transient cotransfection assays, v-*rel* was believed to act as a dominant negative oncogene.

Obviously, one of the important uses of the v-*rel*ER system is the search for genes whose expression is regulated by v-*rel* and v-*rel*ER. This approach should help to identify genes that may be directly involved in transformation. Furthermore, the identification of such *rel*-regulated genes should provide the reagents to study in more detail the mechanism by which v-*rel* interacts which specific target sequences and how it affects, as an activator or repressor, the expression of specific genes.

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ANIMAL HOUSE

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Technician	Gerhard JURITSCH
Technician	Mijo DEZIC
Technician	Erika PENNINGER
Technician	Ryzard KUPINSKI (since October)

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

Current production of animals bred in-house is as follows: approx. 325 mice/week, 50 chicken embryos/week and 10 chickens/week. The *Xenopus laevis* toads bred during 1991 provided sufficient numbers for experimental procedures. For economic reasons, rabbits are purchased from external suppliers.

The animals listed above service the following studies: various mouse projects (see Reports of Erwin Wagner, Denise Barlow, Meinrad Busslinger, Andreas Weith and Max Birnstiel), chicken leukemia studies (see Reports of Hartmut Beug and Martin Zenke) and *Xenopus laevis* studies (see Report of Max Birnstiel).

Technical procedures offered by the animal house are performed to a high standard, thereby ensuring that any discomfort to the animal is kept to a minimum. These include collection of rabbit and chicken blood, collection of mouse sperm, administration of hormones to mice, tumor implantations on mice and chicks, hysterectomy and subsequent resuscitation of potential transgenic and chimaeric mice, collection of embryonic and adult tissues for DNA and RNA analysis.

At the latter end of this year, we took over our designated chicken facility within the University Bio-Center. This facility will maintain 3 breeding flocks enabling us to provide our own fertilized eggs and more chickens for experimental procedures.

The number of scientists requiring mice and technical expertise has increased considerably, therefore, an additional technician has been employed to cover both the "new" chicken facility and the extra work load generated by the additional experimental mice.

COMPUTER GROUP (Protein Modelling)

Anton BEYER	Scientist
Aron BAUER	PhD Student

Introduction

Our main interest is the relationship between amino acid sequence and tertiary fold of globular proteins. We are working on two main projects along these lines.

We are using a relational database system for storing and manipulating structural data of proteins. These data are stored in a compact and consistent way and can be used for protein modelling projects and for statistical analysis.

Atomic coordinates from crystal structures can be used to construct a potential of mean force. Aron Bauer has developed new methods for constructing these potentials. These methods and the use of these potentials for protein structure prediction are described below

Structural Database for Proteins

Anton Beyer

The availability of an easy access to structural information of various kind, like cartesian coordinates, secondary structure assignment based on x ray data or NMR experiments is very important for protein modelling projects. Currently the main source of these data is the Brookhaven Protein Databank (1). The organization of the data in more or less independent datasets for each protein is not very convenient for questions concerning more than one single protein. In the last years several attempts have been made to use the concept of a relational database for addressing these sort of questions (2,3). We are continuing our own work in this direction which has been described in detail last year. At the moment about 300 datasets are already included in our database. Simple geometric checks, like chirality and unusual bondlengths and angles on the original Brookhaven datasets turned out to be important for statistical analysis and can also be used to find an estimate for the overall quality of a certain structure. This has been discussed extensively by Morris et al. (4) These checks are routinely performed on upgrading our database. Inconsistencies in the data are either marked with a flag or completely removed. This is an ongoing effort to keep the structural information in our database as consistent and error free as possible. In the near future we expect a large increase in the amount of data which will become available to the public. Then the question of consistency will become even more important.

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2. Islam. S. A., Sternberg M. J. E. (1989): *A relational database of protein structures designed for flexible enquiries about conformation.* **Protein Eng. 2,** 431-442
3. Huysmans M., Richelle J., Wodak S. J. SESAM (1991): *A Relational Database for Structure and Sequence of Macromolecules.* **Proteins 11,** 59-76
4. Morris A. L., MacArthur M. W., Hutchinson E. G., Thornton J. M. (1992): *Stereochemical Quality of Protein Structure Coordinates.* **Proteins 12,** 345-464

From Fold Recognition to Fold Prediction

Aron Bauer

The use of statistically derived Potentials of Mean Force (PMF) has become a widely accepted new way of describing the stabilising energy of proteins[1]. A common application is the identification of the native fold for a given protein sequence. The most severe limitation arises from the applicability of statistics itself. Only a limited number of protein structures are available, and the imperative of using non-redundant sets of data limits the number of protein chains suitable for statistics to about 150. We suggest the use of common mutation matrices, which have been successfully used in sequence comparison[2], to assist in the construction of the PMF. This method yields smoother and, apparently, more reliable distribution functions for intramolecular distances, which are the basic data for the particular PMF we use. In contrast, additional attempts to improve the PMF, e.g. smoothing, were shown to have only small positive effects (if positive at all).

We used jack-knife tests to assess the PMF's ability to identify the native fold for a given sequence amongst typically thousands of alternative folds[3]. Our refined PMF identifies every protein that can be classified as properly globular. This 'threading' method does not allow for insertions and deletions to occur, when comparing a sequence with a three-dimensional structure. Therefore we set up an algorithm, based on the Needleman-Wunsch scheme, to align sequences against structures. This approach is expected to identify any structure in the database homologous to a given sequence. Such an application might prove useful in sequence analysis and characterisation of (structural) protein families. In contrast to examples in the literature[4] we are using explicit three-dimensional threading and make the alignment feasible by introducing simple rules.

The ultimate goal in every work on protein structure is the complete understanding of structure determining factors and the ability to predict. With a potential that is able to identify the native fold reliably, all that remains is to generate enough trial folds and pick out the native one. Obviously this approach is doomed to fail by the large number of a priori possible conformations for a polypeptide chain. We have chosen a distance geometry description of proteins to be able to incorporate as many constraints as possible (and available) into the model. One prerequisite of model building is a prediction of secondary structure that is fairly accurate. In this context we investigate the predictive power of a screening method, that compares sequences to individual supersecondary structures, and therefore includes long-range interactions, too. Classical secondary structure prediction is confined to the local sequence environment and reaches prediction rates of about 70% in the optimal case, which is not enough for three-dimensional models.

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4. Jones D.T., Taylor W.R., Thornton J.M. (1992): *A new approach to protein fold recognition.* **Nature 358**, 86-89

PUBLICATIONS SUBMITTED 1992

1. A. Beyer, H. Kalchhauser, P. Wolschann (1992): *Molecular Modeling on Garuganin-I.* **Monatsh. Chem. 125,** 417-423
2. G. Casari, A. Beyer: *Measuring the Electrostatic Interaction Potential in Protein Structures.* Submitted 1992

SERVICE DEPARTMENT

Gotthold SCHAFFNER	Scientist
Ivan BOTTO	Technician
Robert KURZBAUER	Technician
Elisabeth LEITNER	Technician (since March 1992)
Andrea SCHNATTINGER	Postdoc (until February 1992)
Christa CZAPKA	Technician Media Kitchen
Gabriele BOTTO	Technician Media Kitchen (since October 1992)

Introduction

The Service Department provides a variety of instant services that are essential for the IMP scientists. Our most popular services are DNA sequencing and oligonucleotide synthesis. In addition to these time consuming services, we also test titers of antisera and discuss immunization protocols with the scientists and the Animal House staff.

Cell culture media and numerous solutions prepared in the Media Kitchen are important for the continuity and reliability of work done in the IMP.

We also prepare and keep stocks of cloning vectors, primers, bacterial strains, including transformation-competent E.coli strains, and various selected reagents like DNA molecular weight markers, enzymes, etc.

Oligonucleotide Synthesis

The average of length of the 1800 oligonucleotides synthesized on an APPLIED BIOSYSTEMS 380 B this year was approx. 27 nucleotides. This indicates an increasing demand for PCR primers and sequencing primers, which reflects the marked increase in sequencing reactions done this year.

Sequencing

We nearly doubled the amount of sequencing reactions this year. 2000+ DNA samples were sequenced the conventional way using the SEQUENASE (USB) protocol with T7 DNA polymerase (PHARMACIA, PROMEGA) and ^{32}P-dATP. In the future we will use exclusively ^{33}P-dATP (NEN DUPONT) because of its higher resolution on autoradiograms and because of safety considerations. About 3000 DNA samples were sequenced with TAQ DNA Polymerase and fluorescent dye labelled dideoxy nucleotides. The DYE DIDEOXY TERMINATOR kit (APPLIED BIOSYSTEMS) for automated sequencing together with the APPLIED BIOSYSTEMS 373 A sequencer, was found to be a very powerfull tool and helped us to substantially increase our sequencing capacity. The sequencer is especially valuable in sequencing large DNAs (> 20 kBP) and PCR products directly.

We have used the same DNAs for automated as well conventional sequencing. There is no major difference in the reliability of sequence data produced from crude DNAs, prepared either by the „boiling prep procedure" or by „alkaline lysis", and DNA isolated from CsCl gradients. The main problems in sequencing double-stranded DNA still arise from „road blocks" for the DNA polymerase, like GpC islands and DNAs with GC contents of 70% and higher. We could not find a general solution to the problem but we are working on it!

PUBLICATIONS

1. Phillips, St., Cotten, M., Laengle-Rouault, F., Schaffner, G., and Birnstiel, M.L. (1992): *Amphibian oocyte and sphere organelles: are the U snRNA genes amplified?* **Chromosoma 101**, 549 - 556.
2. Ellmeier, W., Aguzzi, A., Kleiner, E., Kurzbauer, R., and Weith, A. (1992): *Mutually exclusive expression of helix - loop helix gene and N-myc in human neuroblastomas and in normal development.* **EMBO Journal 11**, 2563 - 2571.

THE PUBLIC RELATIONS OFFICE

Markus Wögerbauer

The official working hours were reduced by half, nevertheless the I.M.P-Public Relations Office continued to provide a full time service.

Activities

The general aim of the PR-Office is to inform the public about the scientific work performed at the I.M.P. in order to remove fears and reservations against genetic engineering among common people. The most effective way to achieve this goal is probably via the mass media. In this respect the Austrian State Television (ORF) was very co-operative. In 1992 three camera teams visited the I.M.P. to interview some of our scientists. Shots were taken from our labs and from the Animal House. A CIS- camera team presented the I.M.P. via local TV-stations to an audience of about 200 million Russian people. The I.M.P. also provided scientific background material for Austrian radio broadcasting programmes.

In order to expose more and more people - especially in our neighbourhood - with aspects of genetic engineering, we tried to make closer our relationship to local newspapers. Many people are afraid of gene technology simply because they have no idea of what we are doing, thus, there is still a lot of educational work to be done.

We also kept in mind to provide information to scientifically trained people - especially to medical doctors. Therefore, we published a series of scientific articles about achievements at the I.M.P in Austrian medical magazines. Nevertheless a large part of the working time is occupied by answering questions of journalists, preparing material for them and by managing grant applications to the Austrian Forschungsförderungsfond for some I.M.P. research groups.

Inauguration of the Vienna Biocenter

The university organized a „Day of Open Doors" to celebrate the opening of the new Vienna Biocenter. The I.M.P. joined the event and offered tours and informational material to interested people.

Visiting Groups

The PR-Office provides infrastructural support for our guests and organizes tours through the I.M.P. In 1992, 32 groups came to visit the I.M.P. 6 of them were high school classes, two groups consisted entirely of high school teachers in biology and chemistry. We also appreciated the visit of the Department of Infectiology and Chemotherapy, Universitätsklinik für Innere Medizin I, AKH Wien. A special seminar was held for students from the Institute of Psychology, University of Vienna. The I.M.P was also frequently visited by students from the medical department of the University of Vienna.

We had several guests from the Austrian Ministery of Science and Research and from the French governmental Health Department.

The PR-Office also supported a student writing a scientific essay about gene therapy for his university entrance examination. Advice for their final examinations and information about the possibilities of further studies at the University of Vienna were given to many other pupils.

I would like to mention Dr. Anton Beyer, Mag. Aaron Bauer, Dr. Gotthold Schaffner, Robert Kurzbauer and Dr. Kurt Zatloukal. Without their excellent help and co-operation tours through the I.M.P. would not have been possible. A big „Thank you!" to all other I.M.P.-members, who supported me in difficult periods.

PUBLICATIONS

1. Wögerbauer, M.: *Gentechnikgesetz - Ja, aber...*
Ärzte Woche Nr. 12 / 1.4.1992, p.17
2. Wögerbauer, M.: *I.M.P. - Qualität verpflichtet*
Ärzte Woche Nr. 23 / 17.6.1992, p. 14
3. Wögerbauer, M.: *I.M.P.-(His)Story*
Ärzte Woche Nr. 23 / 17.6.1992, p. 14
4. Wögerbauer, M.: *I.M.P.-Portrait*
Bender intern Nr. 26 (Juni 1992), p. 7
5. Wögerbauer, M.: *Die Impfung gegen Krebs wird bald Realität*
Ärzte Woche Nr. 36 / 21.10.1992, p. 1
6. Wögerbauer, M.: *Neues Gentransfersystem hilft im Kampf gegen Krebs*
Ärzte Woche Nr. 36 / 21.10.1992, p. 2
7. Wögerbauer, M.: *Impfung gegen Krebs: Traum und Wirklichkeit*
Ärzte Woche Nr. 36 / 21.10.1992, p. 2
8. Wögerbauer, M.: *Verwirklichung eines Traumes greifbar: Impfung gegen Krebs*
Standard-Med / 20.10.1992, p. 1
9. Wögerbauer, M.: *Grundlagenforschung in Österreich*
top medizin 5/92, p. 23

Diploma- and PhD-Theses of I.M.P. Students in 1992

Angelika Amon (Group K. Nasmyth)
Doctorate thesis: *„Characterization of the mitotic kinase in the budding yeast S. cerevisiae"*, submitted to the Universiy of Vienna.

Sylvia Braselmann (Group M. Busslinger)
Doctorate thesis: *„Induction systems and their use for the identification of Fos Target genes"*, submitted to the University of Vienna.

Wolfgang Breitwieser (Group T. Schuster)
Diploma thesis: *„Characterization of a Gene in Yeast Saccharomyces cerevisiae Encoding a Putative Zinc Finger Protein"*, submitted to the University of Vienna.

Oliver Wessely (Group H. Beug)
Diploma thesis: *„Ektopische Expression der a-Kette des humanen GM-CSF Rezeptors in hämatopoietischen Knochenmarkszellen des Huhns"*, submitted to the University of Vienna.

IMP SEMINAR LIST 1992

JANUARY:

02.01.92 **RUDOLF GROSSCHEDL** (UCSF)
„Regulation of lymphoid specific gene expression"
Host: Max Birnstiel

09.01.92 **DIDIER PICARD** (Univ. of Geneva)
„Hormonal regulation of steroid receptor activity"
Host: Meinrad Busslinger

16.01.92 **BRIAN HEMMINGS** (FMI, Basel)
„Protein phosphatase 2A - the other universal Regulator?"
Host: Lisa Ballou

23.01.92 **ENRICO COEN** (John Innes Institute)
„Homeotic genes controlling flower development"
Host: Martin Nicklin

30.01.92 **JOHN TOOZE** (EMBO)
„Tubular early endosomes, a novel endocytic compartment"
Host: Max Birnstiel

FEBRUARY:

06.02.92 **JONATHAN SLACK** (ICRF, Oxford)
„Regional specification in the early *Xenopus* embryo - The role of fibroblast growth factors"
Host: Denise Barlow

07.02.92 **UELI HÜBSCHE** (Univ. of Zürich)
„DNA replication *in vitro*: unexpected lessons from the eukaryotic cell"
Host: Meinrad Busslinger

12.02.92 **ELAINE FUCHS** (Chicago)
„Reverse genetics and transgenic mice lead to a function for intermediate filament proteins and the basis for a human genetic skin disease."
Hosts: G. Wiche and H. Beug

20.02.92 **AMAR KLAR** (NCI, Maryland)
„Fission yeast mating-type switching: Controls of cell lineage, donor selection and donor silencing"
Host: Kim Nasmyth

25.02.92 **JACQUES GHYSDAEL** (Institut Curie, Orsay)
„The c-*ets1* protooncogene product: a transcriptional regulator of a T lymphocyte specific gene"
Host: Hartmut Beug

27.02.92 **ANDREAS GNIRKE** (Washington)
„Transfer of YACs from yeast to mammalian cell"
Host: Max Birnstiel

27.02.92 **RON McKAY** (MIT, Cambridge)
„Differentiation mechanisms in mammalian neuronal stem cells."
Host: Adriano Aguzzi

MARCH:

03.03.92 **ALEXANDER von GABAIN** (Karolinski Institute)
„mRNA in *E. coli* and its possible connection to the heat shock"
Hosts: Rudolf Schweyen and Meinrad Busslinger

05.03.92 **FRITZ MELCHERS** (Basel)
„B lineage-committed stem cells"
Host: Max Birnstiel

10.03.92 **MARTIN HOFMANN** (Karlsruhe)
„The functional relevance of CD44 variation"
Host: Erwin Wagner

12.03.92 **HARALD SAUMWEBER** (Univ. Köln)
„Chromatin proteins in *Drosophila*: functions in vision and courtship behavior"
Hosts: D. Schweizer and M. Busslinger

17.03.92 **PROF. GEORG STINGL**(Vienna)
„Immunoglobulin receptors on epidermal dendritic cells"
Host: Erwin Wagner

19.03.92 **DOMINIQUE STEHELIN** (Institute Pasteur)
„The proto-oncogene c-*ets*-1, angiogenesis and tumor extension"
Host: Martin Zenke

20.03.92 **SYDNEY BRENNER** (MRC)
„Genome analysis"
Host: Kim Nasmyth

26.03.92 **DAVID COSMAN** (Seattle)
„Cytokine receptor families and their use as immunomodulators."
Host: Erwin Wagner

31.03.92 **BRUCE FUTCHER** (Cold Spring Harbor)
„Cyclins and the control of the yeast cell cycle"
Host: Kim Nasmyth

APRIL:

01.04.92 **BORIS MAGASANIK**
„Regulation of gene expression from distant sites in prokaryotes"
Host: Kim Nasmyth

02.04.92 **ROBERT MONTESANO**(Geneva)
„In vitro regulator of capillary and epithelial morphogenesis"
Host: Erwin Wagner

09.04.92 **TONI KOUZARIDES** (Cambridge)
„Interaction between regulatory and general transcription factors: is Rb involved?"
Host: Martin Nicklin

10.04.92 **PHIL SORIANO** (Houston)
„Genetic manipulation of the early mouse embryo"
Host: Erwin Wagner

16.04.92 **JAMES MANFREDI** (Columbia Univ)
„Insights into the role of p53 in the control of normal cell growth"
Host: Adriano Aguzzi

22.04.92 **RUTH LEHMANN** (Cambridge)
„Pattern formation and germline determination in *Drosophila*"
Host: Gustav Ammerer

24.04.92 **ROLF NÖTHINGER** (Zurich)
„Genetic and molecular approaches to sex determination in *Drosophila*"
Host: Max Birnstiel

28.04.92 **SUSAN CARSON** (Molecular Immunology, London)
„Tales of the unexpressed: class II MHC and mouse B cell development"
Host: Erwin Wagner

29.04.92 **MATTHIAS ERNST** (Ludwig Institute, Melbourne)
„Modulation of murine *hck* tyrosine kinase activity by gene targeting in embryonic stem cells"
Host: Erwin Wagner

30.04.92 **BARRY HALL** (New York)
„Selection-induced mutations in bacteria and yeast"
Hosts: IMP PhD Students

MAY:

08.- 10. 05.92	**UNI/IMP CONFERENCE**
14.05.92	**MARKUS NOLL** (Zurich) „Specification of sense organs and neuroblast identities in *Drosophila*" Host: Meinrad Busslinger
21.05.92	**LEE HOOD** (Pasadena) „Immune Recognition, autoimmunity and the genome program" Host: Max Birnstiel
25.05.92	**TONY VICKERS** (UK Human Genome Project) The human genome project and the customers" Host: Andreas Weith

JUNE:

03.06.92	**Jiri Forejt** (Prague) „Genetic analysis of genomic imprinting at the mouse *Tme* locus" Host: Denise Barlow
04.06.92	**ROLF KEMLER** (Freiburg) „From cadherins to catenins: the importance of the cytoplasmic anchorage for epithelial function" Host: Hartmut Beug
11.06.92	**MICK CALLAN** (St. Andrews) „Recent cytological studies on snRNPs in *Xenopus oocytes*" Host: Max Birnstiel
25.06.92	**MARK NOBLE** (Ludwig Institute, London) „Precursor biology, regeneration and tissue repair through precursor transplantation" Host: Erwin Wagner

JULY:

02.07.92	**HANS THOENEN** (Martinsried) „Ciliary neurotrophic factor: promising perspectives for the treatment of degenerative motoneuron diseases" Host: Erwin Wagner
08.07.92	**ULRIK JOHN** (Univ. of Adelaide) „*Drosophila three rows*, an essential gene in mitosis" Host: Léon Dirick
09.07.92	**ROB C. HOEBEN** (University of Leiden) „Towards gene therapy for hemophilia A: vectors for the expression of blood-clotting factor VIII *in vivo*" Host: Max L. Birnstiel
14.07.92	**JIRI KANAKA** (Libechov) „Attempt to knock out CD8b gene in mouse ES cells" Host: Denis Barlow
16.07.92	**JEAN-MICHEL HEARD** (Pasteur Institute) „Using diseased animal models for gene therapy experiments" Host: Max L. Birnstiel
30.07.92	**NANCY HYNES** (Friedrich Miescher Institute) „Receptor tyrosine kinases: their role in normal mammary cell differentiation and as targets for immunotoxins in cancer cells" Host: Erwin Wagner

AUGUST:

13.08.92 **ULI RÜTHER** (EMBL, Heidelberg)
„Functional gene analysis in transgenic mice"
Host: Erwin Wagner

18.08.92 **STEPHEN JOHNSTON** (Dallas)
„Progress with gene gun technology and genetic immunization"
Host: Ernst Wagner

20.08.92 **RENE BERNARDS** (Amsterdam)
„Activation of transcription by *myc* oncoproteins"
Host: Martin Zenke

21.08.92 **DAN LITTMAN** (UCSF)
„Use of transgenic and gene knockout mice to study signals in thymocyte development"
Host: Erwin Wagner

27.08.92 **PAUL ROTHMAN** (Columbia Univ.)
„The molecular mechanisms by which cytokines direct immunoglobulin heavy-chain class-switch recombination"
„Host: Meinrad Busslinger

SEPTEMBER:

02.09.92 **BOB SCHOLTE** (Erasmus Univ.)
„Prospects for gene therapy of Cystic Fibrosis"
Host: Ernst Wagner

03.09.92 **PHILIPPE BR LET** (Pasteur Institute)

Host: Erwin Wagner

04.09.92 **STEVEN SHAK** (Genentech)
„Cloning, expression and activity of human deoxyribonuclease I - a new aerosol treatment for cystic fibrosis and chronic bronchitis"
Host: Matt Cotten

08.09.92 **THOMAS LUDWIG** (EMBL)
„Mannose 6-phosphate dependent transport of lysosomal enzymes"
Host: Denise Barlow

10.09.92 **ROBIN WEISS** (London)
„How retroviruses enter cell"
Host: Erwin Wagner

11.09.92 **LUIS PARADA** (Frederick)
Proto-oncogenes: Important regulators of embryonic development"
Host:Erwin Wagner

17.09.92 **F. LEVY** (Stockholm)
„Early events in the assembly of MHC class I molecules"
Host: Andreas Bachmair

24. - 26.09.92 **IMP RECESS**

OCTOBER:

01.10.92 **ERIC KARSENTI** (EMBL)
„Microtubule dynamics, microtubule motors and spindle assembly"
Host: Hartmut Beug

02.10.92 **ED ZIFF** (New York University)
„Gene regulation by growth factors"
Host: Meinrad Busslinger

02.10.92 **NADIA ROSENTHAL** (Boston)
„Positional specification of myogenic lineages in the mouse embryo"
Host: Erwin Wagner

05.10.92 **MICHAEL GLOTZER** (USCF)
„Cyclin destruction in frogs and yeast"
Host: Angelika Amon

07.10.92 **RICHARD FLAVELL** (Yale Univ.)
„Tolerance, inflammation and autoimmunity in transgenic mice"
Host: Meinrad Busslinger

14.10.92 **JOANNA GRIFFIN** (BiPi, Ridgefield)
„New specific inhibitors of HIV reverse transcriptase"
Host: Hartmut Beug

15.10.92 **GÜNTER KREIL** (Salzburg)
„Studies on hormone-like, opiate and antimicrobial peptides from amphibian skin"
Host: Meinrad Busslinger

16.10.92 **MARTINO INTRONA** (Milan)
„Role of B-*myb* in hematopoietic cell proliferation approached by antisense oligonucleotides"
Host: Martin Zenke

22.10.92 **TAK WAH MAK** (Toronto)
„Normal and disease development in gene targetted mutant mice"
Host: PhD Students

NOVEMBER:

12.11.92 **HOWARD CEDAR** (Jerusalem)
„Molecular mechanisms involved in genomic imprinting"
Host: Denise Barlow

13.11.92 **NORMAN ISCOVE** (Toronto)
„PolyA PCR of hematopoietic precursor cells: a new and powerful approach to the molecular biology of differentiation"
Host: Erwin Wagner

19.11.92 **BERNHARD LÜSCHER** (Hannover)
„Regulation of nuclear oncoproteins through phosphorylation"
Host: Andreas Weith

19.11.92 **ALFRED NORDHEIM** (Hannover)
„Ternary complex factor (TCF): a regulatory target within the c- fos promoter"
Host: Erwin Wagner

26.11.92 **HINRICH GRONEMEYER** (Strasbourg)
„Steroid hormone receptors: Mechanisms of antihormone action"
Host: Martin Zenke

27.11.92 **PAUL VON HOEGEN** (DKFZ, Heidelberg)
„Molecular analysis of signal requirements for the generation of tumors specific immunoresponses"
Host: Thomas von Rüden

DECEMBER:

03.12.92 **FRED CROSS** (Rockefeller Univ)
„STARTING the cell cycle in budding yeast"
Host: Gustav Ammerer

11.12.92 **ROMAN KLEMENZ** (Univ. Zurich)
„Expression of the aB-crystallin and T1 genes in response to stress, oncogenes and growth factors"
Host: Meinrad Busslinger

15.12.92 **ADRIAN BIRD** (Edinburgh)
„Gene silencing by DNA methylation"
Host: Kim Nasmyth

22.12.92 **HEINZ HIMMELBAUER** (Princeton Univ.)
„Clones and maps in man and mouse: PKD-1 and t-complex"
Host: Denise Barlow

I.M.P. PATENT APPLICATIONS 1992

CASE 14/016

"Neuroblastoma-related regulator gene"
Inventors: A. Weith, W. Ellmeier

Continuation of CASE 14/012-14/014

"Viruses from distant species in receptor-mediated gene transfer"
Inventors: M.L. Birnstiel, M. Cotten, Ernst Wagner

Continuation of CASE 14/012-14/014

"New ligands in receptor-mediated gene transfer"
Inventors: M.L. Birnstiel, M. Cotten, W. Schmidt, Ernst Wagner, Kurt Zatloukal

Continuation of CASE 14/014

"Non-viral peptides in receptor-mediated gene transfer"
Inventors: M.L. Birnstiel, M. Cotten, B. Oberhauser, Ch Plank, Ernst Wagner, Kurt Zatloukal

I.M.P. PUBLICATIONS 1992

1. Adams, B., Dörfler, P., Aguzzi, A., Kozmik, Z., Urbanek, P., Maurer-Fogy, I. and Busslinger, M. (1992): *Pax-5 encodes the transcription factor BSAP and is expressed in B-lymphocytes, the developing CNS and adult testis.* **Genes Dev. 6**, 1589-1607.

2. Aguzzi, A., Bothe, K., Anhauser, I., Horak, I., Rethwilm, A., Wagner, E.F. (1992): *Expression of Human Foamy Virus is differentially regulated during Development in Transgenic Mice.* **The New Biologist 4**, 225-237.

3. Aguzzi, A., Ellmeier, W. and Weith, A. (1992): *Dominant and Recessive Molecular Changes in Neuroblastomas.* **Brain Pathology 2,** 195-208.

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INAUGURAL CONFERENCE OF THE VIENNA BIOCENTER

Organized by the Austrian Ministry of Science and Research, by the University Institutes of Biochemistry, of General Biochemistry, of Microbiology and Genetics, of Molecular Biology, of Molecular Genetics and the Research Institute of Molecular Pathology (I.M.P.), Vienna on

"PROTEIN-NUCLEIC ACIDS INTERACTION"

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