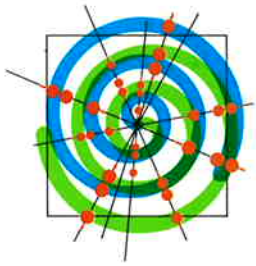


I.M.P.

SCIENTIFIC REPORTS 1989

FOREWORD

Nearly two years have elapsed since the Grand Opening of the I.M.P. on May 26, 1988 and the time has come to take stock of the scientific and organizational developments at the I.M.P. The eightyfive pages of the scientific reports are convincing evidence that the various groups have transplanted their research efforts successfully and, as they tell me, without major delays and this despite the fact that rather complex operations had to be transferred.

Recruitment of the staff was easier than I had feared and today there are 125 employees amongst them 50 PhDs and about 40 diploma and PhD students - a clear case of instant lab. Recruitment was so rapid because once the departmental heads and groupleaders have been selected, they were given the go-ahead to recruit collaborators on their own initiative without interference from the Management. From the abstracts below it will become apparent that we are all geneticists, albeit geneticists of differing persuasions, but the quest for knowledge about gene function, often in the complex biological situations provided by diseases, is the major theme which pervades all our thinking and experimentation.

The concern that the peripheral position in Europe of Vienna would be a disincentive for people to join the I.M.P. was soon dispelled by the recognition of Vienna as a hospitable city with a great many charms and amenities, in fact as one of the great cities of Europe equal to Paris and London. The reception and support of the I.M.P. members by our University colleagues has been exemplary. Cooperation with the Science faculty continues to strengthen our basic science effort, while joint projects with the Medical faculty will increasingly allow us to try out in practical terms the concepts emerging from our scientific studies.

Of course, recent events have changed the geopolitical position of Vienna and what used to be a peripheral position, has proved to be at the centre of a Greater Europe. With great delight one sees that the politicians are very much aware of this new situation and that this has increasingly moved them to "internationalize" Austrian science. Here, too, the I.M.P. can play an important role. At our first two international conferences the participants - 1100 at the inaugural conference and 600 at the second meeting - came almost exclusively from Western Europe; however, it is quite clear from the incoming applications to this year's conference that Eastern European countries will be better represented this time round.

Overall, one can say that luck and serendipity have often been on our side and that the I.M.P. is a success and has become the centre of research excellence envisaged in the contracts drawn up between Genentech and Boehringer Ingelheim in the early Nineteeneighties. Finally, dare I say it, the changeover from an academic life style to working for industry has been easier than most of us expected, one reason being the large degree of freedom in choosing our research topics that the sponsors have generously provided us with. Collaborations with researchers at the sponsor firms have become more frequent as of late, but have probably not reached a sufficient level. Not unexpectedly, we find that scientists at the sponsor firms are deeply entrenched in their own research; to engage them more in cooperative efforts with the I.M.P. is a goal that the research directors should pursue with vigour.

March 12th, 1990

Max L. Birnstiel
Managing Director

SCIENTIFIC REPORTS 1989

RESEARCH INSTITUTE OF MOLECULAR PATHOLOGY

Dr. Bohr-Gasse 7, A-1030 Vienna, fax (222) 78 71 53

Gustav AMMERER	**Group leader**
Michael PRIMIG	**PhD Student**
Jacqueline MERMOUD	**PhD Student**
Beverly ERREDE	**Visitor**

The role of transcriptional control in the mating physiology of the yeast *Saccharomyces cerevisiae.*

Diploidization in the yeast *S. cerevisiae* involves the fusion of two haploid cells with opposite mating type. There are two preconditions for efficient cellular fusion. A) Intercellular communication has to be established. This is accomplished by the selective expression of a complementary set of peptide pheromones and their respective receptors. The decision as to which of the two sets of receptor and pheromone genes will be transcribed depends on the allelic state of the mating type locus. B) The externally received signal must be transduced in order to elicit the appropriate cellular responses: e.g. synchronization of the cell cycle by arresting the cells in G1, morphological changes, and the synthesis of cell-type-specific agglutination factors. One can assume that most of these cellular functions depend on the transcriptional induction of the corresponding genes.

At a first glance it might seem that cell type determination and intracellular signal transduction are functionally related only because one is the precondition for the other. Surprisingly, the two systems are interrelated at the transcriptional level as well. This can be best illustrated by the fact that the integrity of the intracellular signalling pathway is necessary for the transcription of the cell-type determining pheromone and receptor genes themselves. It should be emphasized that this requirement is independent of any externally received signal.

Although these findings have complicated the genetic and molecular analysis of the system, they have also enabled us to gain more information about the signal transduction

pathway simply by studying the requirements for the transcription of cell-type-specific genes. Our principal goal was therefore the identification and characterization of the proteins which bind to the promoter elements of such genes.

The function of the *STE12* product as a transcription factor

The genetic analysis of the mating pathway identified six complementation groups which are essential for mating in both haploid cell types (**a** and α). It was proposed that these genes (*STE4, 5, 7, 11, 12* , and *18*) encode elements of the signal transduction pathway. The DNA sequences which were determined for these genes have been informative for their specific function in some cases. For example, the *STE4* and *STE18* products are similar to the β- and γ-subunits of classical heterotrimeric G-proteins. The predicted *STE7* and *STE11* products share features with known protein kinases. However, no similarities were detected from the predicted STE5 and STE12 proteins. By genetic analysis (e.g. using appropriate dominant activating alleles) it was possible to order the genes within the pathway. Accordingly, STE5, 7, 11, and 12 function downstream of the G-protein complex.

We have defined the function of STE12 by showing that its gene encodes a DNA binding protein. Our conclusions were based on mobility shift assays using *STE12* dependent promoter elements derived from the **a**-factor receptor gene and the transposable element Ty1. For immunological detection of STE12 within the DNA-protein complexes, we tagged the *STE12* gene with a sequence coding for a peptide recognized by a monoclonal antibody. Although we used promoter fragments which normally do not show pheromone induction, it became apparent from DNaseI protection experiments that a sequence called PRE (pheromone response element, TGAAACA) is specifically protected in these STE12 dependent complexes. Previously, this element was shown to mediate pheromone inducible transcription. So far it has been found in at least 2 copies in all promoters that are highly induced by the pheromone. From footprint data we have inferred that STE12 is at least one of the factors which should bind to the PRE sequences in these promoters. It was also possible to show that STE12 binds directly to the PRE sequence using different variants of *STE12* products synthesized in an in vitro system. Under normal conditions the complete

STE12 product will bind only weakly to its target site. However, in two cases we were able to observe increased binding: A) The complete STE12 protein can bind cooperatively with MCM1 when a binding site for this protein is adjacent to the PRE. MCM1 is a protein that is essential for the recruitement of the cell-type-specific regulators α1 and α2. B) A truncated STE12 which lacks two thirds of its carboxy-terminal amino acids can also bind to a single PRE with high affinity. Therefore, the deleted domains seem to exert some negative control over DNA binding. This control may be relieved by the interaction of STE12 with MCM1. If one assumes that STE12 provides the interface with the rest of the transcription machinery, this result could explain why some of the cell-type-specific promoters that seem to rely on *MCM1* function are also *STE12* dependent. The result also has implications for the mechanism of pheromone induced transcription. One can propose two models. In one case the transduced signal modifies the structure of STE12 such that it can now bind more efficiently and cooperatively to the PRE sequences. In another scenario a factor which responds to the pheromone induced signal may become competent to interact with STE12 in an analogous mode to MCM1.

We initiated several approaches to address the question whether STE12 is the recipient of the pheromone induced signal. We used hybrids between STE12 and the DNA binding domain of the GAL4 protein to show that the GAL1-10 promoter became transcriptionally active independent of conditions that normally regulate this promoter. This activation appeared to be dependent on the function of other components of the signal transduction pathway. Another strong indication that STE12 is a target comes from experiments using a 21-bp α-specific promoter element that binds α1 and MCM1 in vitro. We found that the upstream activation function conferred by this fragment is still dependent on the full set of nonspecific *STE* genes. We therefore propose that STE12, MCM1 and α1 can interact on this element even in the absence of a PRE. If so, at least one of these proteins should serve as a target for a pheromone dependent modification, with STE12 as the most likely candidate for it. A more biochemically oriented approach has revealed that dephosphorylation affects the binding of STE12 at least within a complex formed with MCM1. In vivo labelling data also suggest that STE12 is a phosphoprotein. However a direct link

between this modification and the function of the protein as a carrier of the signal is still missing.

What is the role of STE12 in the G1-arrest?

Nonfunctional alleles of *STE12* suppress other dominant mutations which induce the signal transduction pathway constitutively and confer haploid specific lethality. Therefore, we proposed that there should be at least one gene which is specifically involved in G1 arrest whose transcription is dependent on *STE12* function and is not essential for cell survival. In support of this hypothesis we could show that the transcription of *STE4, 5, 7, 11*, and *18* (which are thought to be involved upstream of or at STE12) is unaffected by a *ste12* mutation. In the meantime other labs found mutations in additional genes (called *FAR)* that cause an infertile phenotype. This phenotype may be solely due to the lack of G1 arrest as these mutations do not prevent the induction of other conjugation specific functions. *STE12*-dependent expression of these genes would hint at a central role of STE12 in the different aspects of the pheromone response. In our working hypothesis STE12 would serve as the recipient of the signal induced by the dissociation of the G-protein complex. *STE5, 7,* and *11* identify functions either necessary for STE12 activity or functions that participate directly in the signalling cascade. STE12 would constitute the node from which separate pathways controlling the different cellular responses emerge. In order to test this model we investigated the effect of overproduction of the STE12 protein. An elevated level of STE12 leads to a phenotype which is similar to one found in cells exposed to the mating pheromone: G1-arrest, morphological changes and transcriptional induction of a gene involved in cellular fusion. Although the establishment of these phenotypes is independent of other *STE* products, the cells still appear to be mating incompetent (except for a minor suppression of the mating defect associated with ste7 and ste11 mutations). Therefore STE12 may be sufficient for the induction of the G1-arrest pathway, but it may not be the only factor whose response to the signal transduction pathway is required for a successful completion of conjugation. This is certainly one point we wish to clarify in the near future.

The relevance of the system to gene expression in higher Eukaryotes

The protein encoded by the *MCM1* gene is closely related to the mammalian transcription factor SRF. A bipartite domain of 90 amino acids shows 69 % identity between the two proteins. This domain is necessary and sufficient for the oligomerization and DNA binding in both proteins. SRF has an important function in the immediate early response of mammalian cells to different growth factors. It is also involved in the cell-type-specific transcription of muscle specific actin genes. It has been proposed that SRF has to interact with another protein called p62 in order to mediate the growth factor induced signal. We propose that the mammalian p62 is at least functionally analogous to STE12 if not also related by structure. This is underlined by our finding that the SRF homologous domain of MCM1 is sufficient for STE12 cooperation. Additionally we have shown that the expression of this domain is also sufficient to execute other important functions of MCM1: its involvement in replication, and its activity as a corepressor with $\alpha 2$. Currently we are trying to find out whether hybrid molecules of SRF and MCM1 can substitute for the different biological functions of MCM1.

Publications during the year

Errede, B. and G. Ammerer. 1989. STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. *Genes Dev.* **3**: 1349-1361.

Sorger, P.K., G. Ammerer, and D. Shore. 1989. Identification and purification of sequence-specific DNA-binding proteins. In *A practical approach series. Protein functions* (ed. T. Creighton), pp 199-223. IRL Press, Oxford.

Denise P. Barlow **Groupleader**

Andrea Schnattinger **Postdoc**

Reinhard Stoeger **Ph.D Student**

Kaori Saito **Visiting Student**

Isolation and functional analysis of mammalian developmental control genes from the mouse t complex.

The aim of our research is the isolation and functional analysis of genes, mapping to the mouse t complex, that have a regulatory role in mammalian development and differentiation. We have chosen the mouse t complex, because it is a potential source of a large number of regulatory genes for which naturally occurring mutations already exist, and also because it is one of the best studied and understood genetic systems. The strategy we are using involves three steps. Firstly, high resolution genetic and physical mapping to localize target genes between flanking cloned DNA markers. Secondly, molecular isolation of the delineated genomic region and the identification of a gene. And, thirdly, functional analysis of the target genes using both the wild type and the naturally occurring mouse mutants.

Cloning the genes for *Tme* and t^{w73}

We have used long-range restriction mapping by pulsed-field gel electrophoresis to define the limits of the t^{Wlub2} deletion, that deletes two genes of interest: *Tme* , a maternal transmission effect gene causing lethality during late gestation and most likely representing an "imprinted" chromosomal region and t^{w73} , a recessive lethal gene required during implantation. Our strategy is based on isolating genomic DNA from within this deletion and searching these cloned sequences for candidate genes that have appropriate expression patterns. We have recently

mapped two further genes to this region, one of which manganese superoxide dismutase MnSod, was contained within the t^{Wlub2} deletion (see Figure for details). The biochemical function of MnSOD suggested that it may represent the gene for *Tme*. We have tested this possibility by RNA analysis of mutant embryos and DNA analysis of the genomic MnSOD locus, and it appears that MnSOD is not at the *Tme* locus. The mapping of this enzyme within the t^{Wlub2} deletion has however, identified three t haplotype mutants containing one, three and four copies of the MnSOD gene. Work is continuing to extend the cloned region from within the t^{Wlub2} deletion using the MnSOD gene as a molecular entry point.

Isolation of three novel cDNAs from within the mouse t complex

Molecular isolation of candidate genes regulating male germ cell function: Genes involved in male-specific transmission ratio distortion and sterility have been accurately mapped in the t complex. Transmission ratio distortion is a phenomenon whereby mice heterozygous for the wild type and t form of chromosome 17 (the t form is a naturally occurring chromosomal variant that contains different alleles at many loci), produce morphologically normal sperm that differ in their fertilization efficiency. Sperm carrying the t form of chromosome 17 have an advantage over gametes that carry the wild type, producing a transmission distortion of up to 99%. The molecular basis for this phenomena is not yet known, but genetic analysis has identified two separate components of this system, a responder locus *Tcr* (for which a candidate gene has been cloned, Schimenti et al., Cell 55, p51-58) and three separate distorter loci (*Tcd-1*, *Tcd-2* and *Tcd-3*). Male mice homozygous for the distorter loci are sterile. It is not yet clear whether sterility is a consequence of homozygosity or if the genes responsible for the sterile phenotype are tightly linked to the distorter genes. Following the mapping of an evolutionary conserved random genomic fragment into the region defined as containing the *Tcd-3* locus, we have isolated two tightly linked, testes specific, cDNA clones. Provisional analysis shows that the expression of one cDNA , Tcte-2, is restricted to post meiotic male germ cells and absent in mice that are homozygous for the t form of chromosome 17.

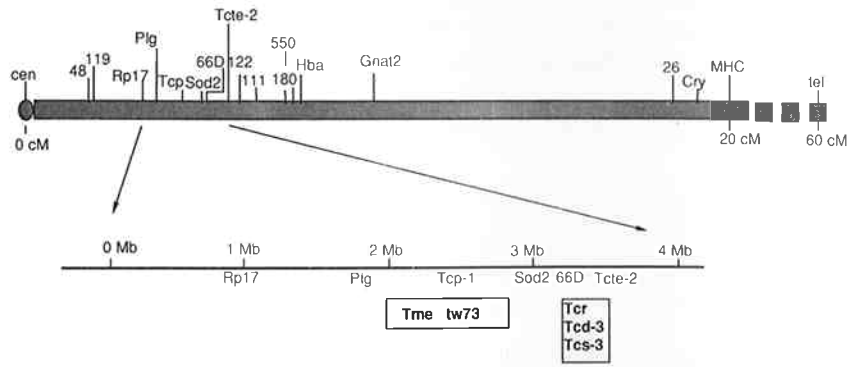
Molecular isolation of a cDNA clone that maps into the t^{h20} deletion. The t^{h20} deletion is located in the distal part of the mouse t complex and contains 5 known developmental control genes. *Fused* and *Fused-kinky/Fused-knobbly* (required for embryonic axial organization), t^0 and t^6 (lethal in the early post-implantation embryo), and *tufted* (required for hair follicle development). We have previously mapped a DNA marker (Tu 12) into this deletion, and have used this, and a second DNA marker already known to map into the same region (Hba-4ps), to generate a long-range restriction map by pulsed-field gel electrophoresis. Our intention was to locate flanking CpG rich islands, and to examine these sequences for evidence of transcription. The pulsed-field gel map identified two CpG rich islands located a maximum of 95kb from the DNA markers. Cosmid clones were identified and used to isolate a cDNA clone from one island. This cDNA clone encodes a 2.2kb transcript expressed in early and late embryonic tissue, and in all adult tissues tested. We intend to continue this approach of finding genes by mapping CpG islands, by continuing the cosmid walk to find the second flanking island.

A genetic map of mouse chromosome 17 comprising 21 DNA markers (In collaboration with Joe Nadeau, The Jackson Laboratory, U.S.)

A high resolution genetic map of mouse chromosome 17 is an essential part of our strategy for cloning genes from the t complex, that have a regulatory role in mammalian development and differentiation. We are in the process of generating a complete genetic linkage map of chromosome 17 using all the available DNA markers. So far, 21 DNA markers have been analysed using an interspecies SPE/BL6 cross (175 animals) and the BxD recombinant inbred panel. Markers that show tight genetic linkage are further analysed by pulsed-field gels for physical linkage and the information used to determine the exact position of target genes relative to the flanking markers. Details of the genomic region containing the *Tme*, t^{w73}, and *Tcr/Tcd-3/Tcs-3* genes are shown in the accompanying Figure.

Genetic and physical mapping of developmental genes to mouse chromosome 17

a) Genetic map: centiMorgans



b) Long-range restriction map in megabasepairs, showing the physical limits of the position of five developmental genes

Use of DNA-intercalating dyes to generate controlled partial digests for pulsed-field gel long-range mapping.

A limiting factor, in the use of pulsed-field gel electrophoresis to obtain chromosome-sized restriction maps, is the length of map that can be generated around any one DNA marker. We have compared the efficiency of producing partial digests by limiting the enzyme concentration, with that obtained by enzymatic digestion in the presence of the DNA intercalating dye ethidium bromide. Both techniques can be used to routinely generate DNA fragments in the 3 - 4 Mbp range, and have been used to map DNA markers from within the t complex.

Publication this year

D. P. Barlow. 1989. Pulsed-field gel electrophoresis. Proceedings XVIth International Congress of Genetics. Genome 31, p465-466.

Hartmut BEUG	**Senior Scientist**
Martin ZENKE	**Group Leader**
Christian SCHROEDER	**Postdoc**
Ernst REICHMANN	**Postdoc**
Christine DISELA	**PhD Student**
Guido BOEHMELT	**PhD Student**
Peter STEINLEIN	**Fellow**
Gabi STENGL	**Technician**
Ursula WALTHER	**Technician**

Oncogene function and cooperation in chicken neoplasms: a potential source of in vitro models for cancer research and therapy ?

Introduction:

Our group is using oncogenic avian retroviruses to study oncogene function and oncogene cooperation in the generation of leukemia. We have mainly concentrated on the avian erythroblastosis virus (AEV) which transforms fibroblastic and hematopoietic cells *in vitro* and *in vivo*. The AEV virus encodes two cooperating oncogene proteins, a mutated epidermal growth factor receptor (v-*erb* B) and a mutated nuclear thyroid-hormone receptor (v-*erb* A). In this system, we try to understand in molecular terms, how these two oncogenes carry out their respective functions, eg. induction of self renewal and hormone independence in erythroid progenitors by v-*erb* B and arrest of erythroid differentiation, probably due to altered gene expression, by v-*erb* A.

Apart from our continuous interest in this particular system, (M.Zenke, C.Disela, C.Schroeder, H.Beug) in which we eventually hope to dissect the various types of changes induced by a cytoplasmic and a nuclear oncogene that together cause a leukemic phenotype in a particular cell type, we have initiated work in two other areas. First, we are trying to utilize avian retrovirus vectors containing transforming, conditionally active (eg. temperature sensitive or hormone-dependent) oncogenes as tools to determine, if and how nontransforming nuclear oncogenes, nuclear hormone receptors and other nuclear proteins involved in transcriptional regulation might alter the differentiation programme of hematopoietic cells after "switching off" the transforming oncogene and thus potentially contribute to a leukemic phenotype (M.Zenke, C.Schroeder and G.Boehmelt). Secondly, we have started to use a mammary epithelial cell system which retains many features of primary mammary gland cells to study the potential effects of oncogenes on processes important for carcinoma formation, eg. loss of differentiated functions, alterations in cell polarity and changes in epithelial/endothelial or cell-matrix interactions (E. Reichmann).

A. c- and v-*erb* A

Towards an understanding of the mechanism how the v-*erbA* oncogene contributes to leukemic cell transformation

Martin Zenke, Christine Disela and Christian Schroeder

(in collaboration with Hartmut Beug, Björn Vennström (Stockholm) and Doug Engel (Chicago))

The v-*erb* A oncogene encodes a mutated version of a nuclear high-affinity thyroid hormone receptor (c-*erb* A) which due to the multiple point mutations and a small C-terminal deletion has lost the ability to bind thyroid hormone but still binds to DNA (Sap et al. 1986). It has previously been established that the v-*erb* A oncogene contributes to the generation of erythroleukemia by blocking differentiation of transformed erythroid progenitor cells and to alter the complex growth requirements of these cells (Kahn et al.1986). We have shown that these phenotypic effects of v-*erb* A are correlated with and probably caused by v-*erb* A-

mediated repression of a set of erythrocyte-specific genes, expression of which is reqired during normal erythrocyte development (carbonic anhydrase II (CAII), the erythrocyte anion transporter (band3) and a key enzyme of heme biosynthesis, δ-aminolevulinate synthase (ALA-S), Zenke et al.,1988 and M.Zenke and H.Beug unpublished).

Recently we have investigated the possible role of the normal thyroid hormone receptor (c-*erb* A) in erythroid cell transformation and/or differentiation, utilizing suitable c-*erb* A-expressing retroviruses. We found that in erythroid cells expressing high levels of exogeneous c-*erb* A, erythrocyte differentiation is repressed in the absence of thyroid hormone (T_3) but activated after addition of T_3 (Zenke et al. in preparation). In these cells, transcription of those erythrocyte-specific genes which are repressed by v-*erb* A (CAII, band 3, ALA-S) was regulated in a hormone-responsive fashion.(i.e suppression in absence, but induction in presence of hormone). This suggests that the v-*erb* A oncoprotein has lost one type of normal receptor function (i.e. to induce transcription in the presence of hormone), but constitutively displays another function (i.e. that of a repressor of transcription in the absence of T_3).

Pilot experiments designed to determine, which of the *erb* A-affected genes would be subject to direct transcriptional control by v- and c-*erb* A prompted us to focus on the carbonic anhydrase II (CAII) gene. By immunoprecipitation of v-*erb* A-DNA complexes using anti-*erb* A antibodies we have identified potential v-*erb* A binding sites in upstream promoter sequences of the CAII gene. In vitro footprinting showed that this v-*erb* A recognition site also bound the normal thyroid hormone receptor c-*erb* A and exhibited sequence motifs characteristic for thyroid hormone responsive elements (T_3REs). Transient expression experiments are in progress which should establish whether this *erb* A binding site in the CAII promoter constitutes a bona fide thyroid hormone responsive element and confers thyroid-hormone responsiveness to a heterologous promoter .

v-*erb* A arrests differentiation of normal erythroid progenitors without inducing sustained self renewal

Christian Schroeder, Ulrike Fuhrmann and Hartmut Beug

If one of the primary effects of the v-*erb* A oncogene is to cause a differentiation arrest in erythroid cells, v-*erb* A should do so even in absence of another transforming oncogene.To approach this we have introduced the v-*erb* A oncogene into normal erythroid progenitors using a v-*erb* A retrovirus containing the neomycin resistance gene as a selectable marker. We found, that v-*erb* A caused an outgrowth of immature, erythroblast-like cells from infected bone marrow. These v-*erb* A infected erythroblasts ceased to proliferate and disintegrated after 9 to 18 divisions while erythroblasts transformed by tyrosine kinase oncogenes like v-*erb* B or v-*sea* grew for 25 to 50 population doublings and showed distinct signs of senescence before cell death. The v-*erb* A infected erythroblasts exhibited a significant suppression of CA II and band 3 gene transcription and required erythropoietin for survival. These results suggest, that v-*erb* A arrests differentiation in early erythroid progenitors without grossly altering their limited ability to proliferate in response to erythroid growth factors. This conclusion was confirmed by a detailed analysis of several v-*erb* A/ts v-sea transformed erythroblast clones which exhibited a very similar behaviour when their transforming kinase oncogene was switched off by cultivation at the nonpermissive temperature for several days.

B. Effect of retinoic acid receptor and steroid hormone receptors on erythrocyte differentiation and gene expression

Christian Schroeder, Martin Zenke and Hartmut Beug

Recent results by others (Umesono et al.,1988) indicate, that the thyroid hormone receptor and the retinoid acid receptor are highly homologous in the DNA binding region and recognize the same (artificial) hormone response element. Therefore, we compared the effects of thyroid hormone and retinoic acid on temperature-induced differentiation of ts-oncogene transformed erythroblasts. Since only the latter compound had a significant effect in this system, we have initiated a project to introduce the retinoic acid receptors (RA^R type a and ß) as well as the more distantly related estrogen receptor into hematopoietic cells via retrovirus mediated gene transfer and to investigate their effect on erythrocyte differentiation

and gene expression. In addition,chimeric genes encompassing the DNA binding regions of the different receptors plus the mutated hormone binding region of v-*erb* A are being constructed. These experiments should reveal whether the retinoic acid receptor affects expression of a similar or different set of genes than the thyroid hormone receptor c-*erb* A, whether overexpression of these receptors affects their target gene specificity and whether any of these receptors could become oncogenic in a mutated form.

C. Receptor-type oncogenes

Transformation of hematopoletic cells by the v- and c-*fms* oncogenes

Ulrike Fuhrmann and Hartmut Beug, in collaboration with B.Vennström (Stockholm) and T.Tamura (Giessen, FRG).

Studies initiated at the EMBL (see EMBL research report) had demonstrated, that an avian retrovirus correctly expressing the v-*fms* oncogene induced erythroid progenitor cells to self-renew in an erythropoietin-independent manner. Spontaneous differentiation of the transformed cells was arrested by v-*erb*A. More recent work established, that v-*fms* failed to induce tranformation of myeloid cells, but caused *myb*-transformed myeloid cells as well as normal macrophages to proliferate independently of cMGF (chicken myelomonocytic growth factor, Leutz et al, 1984). Unlike other tyrosine-kinases, v-*fms* did not induce cMGF secretion in *myb*-myeloblasts, suggesting a non-autocrine mechanism of growth factor independence. In a different set of experiments, a complete cDNA clone encoding a receptor highly homologous to the mammalian c-*fms* gene has been isolated and characterized (see EMBL research report 1988). Antibodies raised to the bacterially expressed protein demonstrated, that this putative chicken c-fms gene is expressed at the surface of chicken macrophages but not on erythroid cells. In collaboration with T. Tamura, we are currently introducing this gene as well as mammalian/avian chimeric *fms* genes into chicken retroviruses to analyse their potential effects on erythroid and myeloid avian cells.

The human EGF receptor causes ligand-dependent abrogation of growth factor dependence and induction of maturation in v-*myc*-transformed avian macrophages

Martin Zenke and Hartmut Beug

Studies initiated at the EMBL had shown, that macrophages transformed by a retrovirus expressing v-*myc* together with the normal human EGF receptor could be cultivated in EGF instead of avian hematopoietic growth factors such as cMGF (Leutz et al,1984). In addition, high doses or prolonged cultivation of the transformed macrophages in EGF induced them to mature and/or to become activated (as demonstrated by withdrawal from cell cycle, increased expression of lysozyme, fibronectin and macrophage differentiation markers and reduced expression of transferrin receptor). We are planning to utilize this system to test mutant EGF receptors, other growth factor receptors and proteins that modulate the macrophage phenotype.

Transformation of differentiating mammary epithelial cells by growth factor receptors and related oncogenes.

Ernst Reichmann, in collaboration with Thomas von Rüden (IMP) and Roel Nusse (Amsterdam)

In previous work, a nontumorigenic mouse mammary cell line could be established, that synthesized large amounts of β-casein upon stimulation with lactogenic hormones. Cloning of this cell line yielded fibroblastic and epithelial sublines, that were unable to produce casein on their own. Upon cocultivation of epithelial and fibroblastic cells, however,characteristic structures resembling inside-out glandular ducts were formed, in which a laminin-containing basal membrane was deposited at the border between the two cell types and in which the epithelial cells could be induced to produce casein and cytokeratins at high levels (Reichmann et al,1989).

At the IMP, we have started to introduce normal and mutated growth factor receptors into these cells, trying to answer the question, how oncogenes might interfere with structural and functional differentiation of mammary epithelial cells.

D. Other projects:

Transferrin-polylysine conjugates as a means to introduce DNA into hematopoietic cells.

Martin Zenke, Peter Steinlein and Hartmut Beug in collaboration with Ernst Wagner, Matt Cotten and Max Birnstiel (IMP)

Extensively proliferating cells require large amounts of iron and therefore express high levels of transferrin receptor on their cell surface. We tested whether the transferrin cycle could be used to introduce DNA into hematopoietic cells via receptor-mediated endocytosis. This method might allow to introduce and express anti-sense RNA or ribozymes in leukemic cells, that specifically neutralize oncogene activities involved in transformation of these cells (see report of Max Birnstiels group).

Since differentiating erythroid cells exhibit a particularly active transferrin cycle, we have assayed various transferrin-polylysine (TfpL) conjugates (see report by Ernst Wagner) for their ability to replace native avian transferrin in erythroid differentiation (Schmidt et al,1986). All conjugates were able to mediate the extensive iron transport required for erythroid differentiation, although with a somewhat lower specific activity. This was true also for TfpL conjugates complexed to DNA.

In the next step, we monitored the efficiency of gene transfer into erythroblasts by TfpL-conjugates, using plasmid DNA encoding firefly luciferase under control of the Rous Sarcoma Virus (RSV) LTR. Under optimized conditions, the RSV-luciferase plasmid could be transferred into both established erythroid cell lines and primary erythroid cell clones, albeit with an efficiency about 80 to 100 fold lower than that obtained with DEAE-dextrane transfection (Choi and Engel,1988), a technique which is very efficient, but also quite toxic

(killing 30 -50 % of the transfected cells). DNA transfer by TfpL-conjugates was shown to be specific and dependent on the transferrin cycle, since no DNA transfer was obtained using either transferrin-DNA or polylysine-DNA mixtures. In addition, free transferrin added in excess strongly inhibited DNA transfer by the TfpL- conjugates.

In pilot experiments, we have also used TfpL-conjugates to introduce t-RNA-ribozymes targeted to the v-*erb* B oncogene (see report of Matt Cotten) into v-*erb* B transformed erythroblasts. These cells were then analyzed for an enhancement of spontaneous differentiation, which would indicate a suppression of the transformed phenotype. Although small, but specific increases in the number of differentiating cells were seen, attempts to increase the efficiency of v-*erb* B suppression were unsuccessful so far. We are currently trying to enhance the efficiency of gene transfer.

Publications 1989

Frykberg,L., Metz,T., Brady, G., Introna,M., Beug,M., Vennstroem, B and Graf, T.(1988).A point mutation in the DNA binding domain of the v-*myb*-oncogene of E 26 virus confers temperature sensitivity for transformation of myelomonocytic cells. Oncogene Res. **3,** 313-322.

Graf,T., Kahn,P., Leutz,A., Beug,H., and Vennstroem, B.(1988) Cooperativity of viral oncogenes in avian leukemia. In: Cell differentiation, Genes and Cancer, Kakunaga,T, et al., eds. IARC Scientific publications **92,** pp165-171.

Vennstroem,B., Sap,J., Schmitt,J., Forrest,D., Munoz,A., Zenke,M., Stunnenberg,H., Beug,H. (1989) The steroid/thyroid hormone receptor family and gene regulation. In: Life Sciences Birkhaeuser Congress Reports, Carlstedt-Duke,J., Eriksson,H. and Gustafsson,J.-A., eds. Birkhaeuser Verlag Basel, pp161-168.

Vennstroem,B., Beug,H.,Forrest,D., Johnsson,A., Khazaie,K., Munoz,A., Sap,J., Ullrich,A. and Zenke,M. (1989) Functions of the erb A and erbB oncogenes in avian erythroblastosis. NATO ASI series Vol H 26,Cell to cell signals iin mammalian development, D.de Laat, ed., Springer Verlag Berlin, Heidelberg, pp289-300.

Beug. H., and Graf, T. (1989) Cooperation between viral oncogenes in avian erythroid and myeloid leukemia. Europ. J. Clin Invest., in press.

Fuhrmann,U., Vennström B., and Beug,H. (1989) Lineage specific transformation of chicken hematopopietic cells by the v-fms oncogene. Genes and Development **3**, 2072-2082.

Forrest, D., Munoz, A., Raynoshek, C., Vennstrom, B. and Beug, H. (1989). Requirement for the C-terminal domain of the v-erbA oncogene protein for biological function and transcriptional repression. Oncogene, in press.

Other references:

Choi and Engel (1988) Cell **55**,17-26
Kahn et al. (1986) Cell **45**,349-356.
Leutz et al. (1984) EMBO J. **3**, 3191-3197
Reichmann et al. (1989) J Cell Biol **109**, 1127-1138
Sap et al. (1986) Nature **324**, 635-640
Schmidt et al. (1986) Cell **46**, 41-51
Umesono et al. (1988) Nature **336**, 262-265.
Zenke et al. (1988) Cell **52**, 107-119.

Anton BEYER — **Scientist, Group Leader**

Georg CASARI — **PhD Student**

COMPUTING, PROTEIN MODELLING

Modelling of Homologous Proteins: Rhinovirus 2A Protease

Most successful examples in protein structure prediction so far are cases where the 3D-structures of homologous proteins have been solved. In these cases the known structures may be taken as a template on which the new ones will be modelled. Here a good alignment of the sequences is crucial for useful results.

It has been found that structural features are much better conserved than the sequence. This justifies the attempt to take the structure of a far related protein as a template. In these cases the proper alignment of the sequences and right positioning of gaps becomes difficult. As homology gets poor an unambiguous alignment cannot be achieved by standard techniques. Secundary structure prediction may help. Very often a decision can be made only in going back and forth between the alignment of the sequence and modelling on the 3D-structure.

In November 1988 Bazan and Fletterick published a faint but significant homology and an alignment between the 2A proteases of picornaviruses (a type of cysteine proteases) and the trypsin-like family of serine proteases[1]. With this knowledge in mind we tried to align the sequences of rhinoviral 2A proteases with small trypsin-like proteases. At the current state we got a model that resembles Bazan's model in general but has some substantial differences. In a close collaboration with W. Sommergruber's group (BENDER company) and E. Küchler's group (Universität Wien) who have the rhinoviral system under investigation we try to improve and confirm that model with experimental data.

Calculations on Proteins: Energy potentials

All calculations on protein structures use some kind of Energy potentials for the proteins. The most widely used potentials are based upon modelling the aminoacids atom by atom. These potentials have failed to give a useful means to evaluate or predict a structure of a protein due to two reasons:

1. All simplifications (like calculations in vacuum) lead to unrealistic and unphysiological conditions and might at their best give estimations of a protein under these strange circumstances.

2. Use of these potentials for molecular dynamics, energy minimisations, Monte Carlo calculations ... require an enormous computing power but can still simulate only processes in the range of psecs (10^{-12}) to nsecs (10^{-9}). This is far from the fastest enzyme katalyzed reactions and several orders of magnitude away from processes like protein folding (msecs to minutes).

Recently M. Sippl (Universität Salzburg) has developed a fully novel concept to deduce energy potentials from known X-ray structures(2). In November 1989 we started a close collaboration with him. We try to systematically establish and test the methodology and the potentials.
First tests on well known problems of protein structure prediction look extremely promising (see (2)).

Bibliography:

(1) Bazan J.F. & Fletterick R.J.; Viral cystein proteases are homologous to the trypsin-like family of serine proteases: Structural and functonal implications; (1988) Proc. Nat. Acad. Sci. USA 85, 7872-7876.

(2) Sippl M. ; The Calculation of Conformational Ensembles from Potentials of Mean Force; accepted (Dec 1989) Journ. of Mol.Biol., in press.

Adrian BIRD	**Senior Scientist**
Francisco ANTEQUERA	**Postdoc**
Richard MEEHAN	**Postdoc**
Jamal TAZI	**Postdoc**
Joan BOYES	**PhD Student**
Joe LEWIS	**PhD Student**
Elke KLEINER	**Technician**
Eugen ULRICH (since April 1989)	**Diploma Student**

Genome Structure, Organisation and Modification

This group is interested in understanding how the structure and organisation of the mammalian genome affects the ability of the transcription machinery to recognise those DNA sequences that are to be transcribed. The problem arises because nuclei contain a very large amount of DNA (about 3 metres per nucleus in humans), and because the genes represent a tiny minority of this DNA (a few per cent). Does the transcription machinery interact with the entire mammalian genome in its search for DNA sequences that fulfil the preconditions for triggering productive RNA synthesis, or are there structural cues which make selected areas of the genome especially accessible in the nucleus?

A structural feature that distinguishes DNA containing genes from the rest concerns DNA methylation. Mammalian gene promoters are often within discrete sequences that are rich in non-methylated CpG (the so-called "CpG islands"). Bulk DNA, on the other hand, contains a lower density of CpG, and this is mainly in a methylated form. A major goal of the laboratory has been to understand the significance of this difference. One approach was to

look for proteins that distinguish island-like from non-island-like DNA (J. Lewis, R. Meehan). A second approach was to ask whether CpG islands and bulk DNA are differentially organised in the nucleus. This we have done in two ways: (i) by probing the nuclease-sensitivity of methylated and non-methylated CpGs in the nucleus (F. Antequera); (ii) by isolating and studying CpG island chromatin (J. Tazi). In another series of experiments, we have begun to analyse in detail the mechanism by which DNA methylation leads to the suppression of transcription (J. Boyes). Not all the results of these various approaches are yet in, but the sum of evidence so far suggests a coherent model to explain the effects of DNA methylation in the genome. Our future intention is to test this model more rigorously. This report will describe the above work, and will also outline more recent studies of unscheduled methylation of CpG islands in cultured cell lines, and the consequences of that.

Inaccessibility of methyl-CpG sites in the nucleus

F. Antequera, D. Macleod (Edinburgh)

If the presence of methyl-CpG in bulk DNA, and its absence in CpG islands, somehow leads to a difference in chromatin structure at these two kinds of DNA, we would expect to detect this difference using nuclease-sensitivity as an assay. The nucleases that we used were MspI (recognising CCGG) and TthI (recognising TCGA). Both enzymes cleave at CpG, and both are normally indifferent to the presence of CpG methylation. In nuclei, however, the majority of methyl-CpGs are protected against MspI and TthI digestion, whereas non-methylated CpGs in the same nuclei are accessible (Antequera et al, 1989).The finding that both enzymes behave as though they are methyl-sensitive when intact nuclei are the substrate, implies that a factor (e.g. a protein) is specifically associated with methyl-CpG. That methyl-CpG itself is the likely target of protein binding was indicated by the finding that non-CpG sites in the same genomic regions were sensitive to nuclease digestion (Antequera et al, 1989).

Proteins that bind to methylated DNA

J. Lewis, R. Meehan, E. Kleiner, S. McKay (Edinburgh)

We have identified proteins that could be involved in mediating the nuclease-resistance of methyl-CpGs in nuclei (Meehan et al, 1989). The proteins, known as Methyl-CpG Binding

Proteins (MeCPs), are able to bind to a wide variety of DNA sequences provided they contain multiple methyl-CpGs. The proteins were detected by incubating nuclear extracts with short oligonucleotide duplexes that were either methylated or non-methylated. Complexes were then detected either by band-shift or South-Western assays. Binding is tolerant of variable distances between methyl-CpGs, and of variable intervening sequences. We are in the process of purifying MeCPs in order to study their structure, localisation in the cell, abundance, interaction with chromatin, and effects upon transcription of methylated genes.

MeCPs are easily detected in most, but not all cell types. The exceptions are two embryonal carcinoma cell lines and an embryonal stem cell line, all of which have very low MeCP activity. Methyl-CpGs in these nuclei showed significantly increased sensitivity to nucleases, in keeping with the hypothesis that MeCPs may be responsible for their nuclease insensitivity in other cell types (Antequera et al, 1989).

CpG Island chromatin

J. Tazi

Much is known about chromatin organisation at the nucleosomal level, but there is little conclusive information about higher order folding of nucleosomes, or about their unfolding during transcription. As mentioned above, an MeCP-like protein may be involved in higher order chromatin folding to give a structure that is incompatible with transcription. In contrast, CpG islands contain the 5' domains of many genes, and therefore represent transcriptionally active chromatin. Due to the nuclease-resistance of methylated CpGs, we have been able to selectively release CpG island chromatin from nuclei and study its composition (Tazi and Bird, submitted). These experiments for the first time provide an opportunity to study an active chromatin fraction from mammalian cells at the biochemical level. Initial results show that CpG island chromatin differs markedly from bulk chromatin in several respects:

(i) Histones H3 and H4 are highly acetylated.

(ii) Histone H1 is greatly reduced in amount.

(iii) A large fraction of the DNA is nucleosome-free.

We intend to study the fraction further to establish its other properties. For example: the extent and localisation of the nucleosome-free regions; the involvement of HMGs 14 and 17 in the active structure; the structure of chromatin at transcriptionally inactive islands.

How does DNA methylation inhibit transcription

J. Boyes

The inhibitory effects of DNA methylation on gene expression have received considerable attention, but the mechanism of inhibition is not yet fully understood. The simplest potential mechanism involves *direct* interference of methyl-CpG with the binding of transcription factors, and there is evidence that some factors are indeed prevented from binding to methylated sites. Another potential mechanism involves the interaction of methylated DNA with nuclear components leading to a distinctive structure that is incompatible with transcription. Inhibition would in this case be *indirect*. Several lines of evidence are compatible with the indirect mechanism. We set out to investigate the question systematically, initially by comparing transcription of a methylated gene (the human alpha globin gene) in *in vitro* extracts and in transient transfection experiments. Results so far show that inhibition *in vitro* is negligable under normal conditions, but is strong in cells using the same template. Absence of inhibition in vitro would not be expected if DNA methylation prevented access by transcription factors directly. The result therefore provide preliminary support for indirect inhibition in this case. Our intention is to extend this analysis considerably, and, if possible, to link it to the studies of MeCPs that are proceeding concurrently (see above).

Unscheduled methylation of CpG islands in permanent cell lines

F. Antequera, J. Boyes

There is now a body of evidence that low frequency *de novo* methylation of CpG islands may play a causal role in the long term inactivation of genes in culture. The phenomenon has sometimes been seen as equivalent to "gene regulation" in cultured cells, but its characteristics are much more analogous to mutation, since it leads to heritable repression of transcription. Indeed, many of the loss-of-function variants in cultured cells that were originally designated as mutants are in fact reversible by treatment with the methylation-inhibitor 5-azacytidine and are associated with methylation of CpG island sequences. Holliday (1988) has proposed the term "epimutation" to describe heritable variations of this kind that do not involve changes in DNA base sequence. We were interested to determine the extent of CpG island methylation in permanent cell lines of mouse and human origin, and to understand the

mechanism by which it could be involved in transcriptional repression. We focussed initially on tissue-specific genes that are probably non-essential in culture. Changes in chromatin that might prevent expression of these genes should have no effect on the phenotype of the cells. The experiments showed that methylation of CpG islands is surprisingly widespread at genes of this type, and correlates with changes in the structure of island chromatin that are equivalent to those seen during X-inactivation. In contrast, de novo methylation was not seen at the CpG islands of housekeeping genes, as predicted if methylation-mediated repression of these genes is incompatible with survival of the cells. The difference between housekeeping and tissue-specific genes was not due to immunity to de novo methylation of housekeeping genes, since selection against their expression has been shown to result in methylation-mediated repression. The results lead to the conclusion that the selectivity of DNA methylation which insures that CpG islands remain methylation-free in cells of the animal are significantly relaxed in many cultured somatic cells. Progressive inactivation of CpG island-associated genes by de novo methylation apparently leads to the long term suppression of genes that are non-essential in culture, or, in the case of housekeeping genes, to cell death. Epimutations of this kind may account for the well known loss of differentiated functions in cultured cell lines derived from specific tissues.

Frequencies of "rarecutter" restriction sites in CpG islands

A. Bird

Our earlier finding that non-methylated sites for rare-cutting restriction enzymes are concentrated in CpG islands has helped in the construction of long range maps of mammalian DNA, and in the search for genes. That work was based on calculation of the *expected* frequencies of rarecutter sites in CpG islands. There are now sufficient CpG island sequences in the data base for a realistic estimate of the *observed* frequencies of these sites. An analysis of 19 CpG island sequences (Bird, 1989) showed that most rarecutter sites occur at the expected frequency. Some, however, are significantly rarer than expected and will therefore cut most often outside of CpG islands. This finding has implications for the design of long-range mapping experiments.

The expected equilibrium of the CpG dinucleotide under a mutation model

Collaboration with J. Sved (Sydney, Australia)

The CpG di-nucleotide is present at about 20% of its expected frequency in mammalian genomes, a finding that is thought to be due to a high rate of mutation from the methylated form of CpG to TpG and CpA. We have examined the hypothesis that the 20% frequency represents an equilibrium between the rate of creation of new CpGs and an accelerated rate of CpG loss due to methylation. On the basis of this model we calculated the expected reduction in the equilibrium frequency of the CpG di-nucleotide, and found that the observed CpG deficiency could be explained by mutation from methyl-CpG to TpG/CpA at about 20 times the normal transition rate, the exact rate depending on the ratio of transitions to transversions. The observed rate of loss of CpG di-nucleotides in a human a-globin non-processed pseudogene, Ya1, and the apparent replenishment of the CpG pool in this sequence by new mutations, were in agreement with the above parameters. We used these figures to estimate that derivation of the present pseudogene sequence from a methylated form of the a1 sequence would be accomplished in 12-25 myr.

Publications during 1989

Antequera, F., Macleod, D. and Bird, A.P. (1989) Specific protection of methylated CpGs in mammalian nuclei. Cell **58**, 509-517.

Meehan, R.R., Lewis, J.D., McKay, S., Kleiner, E.L. and Bird, A.P. (1989) Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell **58**, 499-507.

Bird, A.P. (1989) Two classes of observed frequency for rare-cutter sites in CpG islands. Nucl. Acids Res. **17**, 9485.

Bird, A.P. and Tazl, J. (1989) Alternative chromatin structure at CpG islands. Submitted

Sved, J. and Bird, A.P. (1989) The expected equilibrium of the CpG dinucleotide under a mutation model. Submitted

Max L. BIRNSTIEL	**Managing Dir./Group leader**
Matt COTTEN	**Postdoc**
Ingrid HOFFMANN	**Postdoc**
Francoise LÄNGLE-ROUAULT	**Postdoc**
Richard ECKNER	**PhD-student**
Harald KANDOLF	**PhD-student**
Karim TABITI	**PhD-student**
Alain VASSEROT	**PhD-student**
Birgit MANDL	**Diploma student**
Wilfried ELLMEIER	**Diploma student**
Karin KOS	**Technician**
Bruce SELLS	**Visiting Professor**

Work in this group is divided equally between studying on the one hand the regulation of histone mRNA metabolism, inclusive of the **regulation of 3' processing** and of **histone mRNA stability** and on the other hand developing new means to inhibit target sequences within the living cell by means of ribozymes and antisense oligonucleotides. For this latter topic we have adapted cellular **receptor mediated endocytosis mechanisms** for the import of oligonucleotides and DNA.

The specific inhibition of genes afforded by oligonucleotides relies on the ability of such complementary RNA or DNA sequences to hybridize to mRNAs or to processing signals of pre-mRNAs and in this way to stop the information transfer from specific genes to proteins. In the case of complementary RNA oligonucleotides, hybrid arrest of translation or processing ensues, RNA/RNA hybrids representing a structual impediment for translation or processing.

An interesting variation of the complementary RNA approach is the use of complementary RNAs which are ribozymes. Such "hammerhead" ribozymes can be targeted to cleave specific sequences within virtually any RNA. The ribozymes have the great intrinsic advantage of being catalytic entities: i.e., once a sufficient cellular internal pool has been created, they should, at least in theory, promote cleavage and inactivation of pre-mRNAs and mRNAs without at the same time being consumed themselves.

Two crucial problems confront the experimentor:

a. the poor penetration of highly charged oligonucleotides into the cell and

b. the biodegradation of the oligonucleotides or ribozymes.

In order to circumvent these problems various other groups have developed oligonucleotides which in some cases are more electroneutral (and therefore penetrate the cell membrane more easily) and at the same time are chemically modified in such a way that cellular enzymes cannot act on them.

Despite difficulties, some spectacular results have been obtained, albeit mostly in model systems, either by anti-mRNA produced from within or by incubating tissue culture cells with complementary oligonucleotides. Striking examples from other labs are: the Shiverer syndrome has been reconstructed in transgenic mice by expressing anti-BMP mRNA; potato plants expressing antiviral sequences have been rendered immune against viral infection (at low multiplicity of infection). Sturdier tomatoes have been generated using antisense polygalacturonase genes. The propagation of HIV virus has been inhibited in tissue culture cells using oligos.

The molecular mechanisms that lead to the synthesis of histone mRNAs provide a suitable model system for us to learn how to improve on the various parameters of the antisense and ribozyme technique. Rapidly proliferating cells synthesize a distinct set of histones in step with DNA, the so-called replication variant histones. Cells that have ceased to divide and have become terminally differentiated replace a portion of the replication variant histones with the so-called replacement histone variants over a period of weeks and months. In addition, in some species like the sea urchin, there is a developmental program for replication variants, those of the blastula differing from those of the gastrula and later developmental stages.

The question has remained unanswered whether such variation in histone structure is functionally important or, whether, in genetic terms, there will be a phenotype if the replication variants are suppressed. All known replication variant histone genes have a common denominator in that their pre-mRNAs are processed in a reaction involving the U7 snRNP, whose 5' sequences basepair with a conserved spacer sequence of the histone pre-mRNA. These 5' sequences of the U7 are a suitable target for testing the antisense/ribozyme approach.

For medical applications the inefficient uptake of nucleic acid molecules into cells presents a substantial limitation to the inhibition of genes by antisense oligonucleotides, by ribozymes or to the introduction of artificial gene constructs. To overcome this barrier our efforts aimed at the adaptation of natural transport systems such as receptor mediated endocytosis mechanisms for the delivery of natural or modified oligonucleotides as well as for the delivery of high molecular weight DNA. The inhibition of U7 RNA mediated 3' processing of histone pre-mRNA is one research goal in this area, to develop methods to inhibit HIV propagation another.

MOLECULAR MECHANISMS OF HISTONE mRNA 3' END FORMATION.

A. Vasserot and K. Tabiti

There is, for a proliferating cell, an obviously vital need for histones during the S phase of the life cycle. To meet this requirement, histone gene expression is controlled at multiple levels in such a way that the abundance of the histone mRNAs closely parallels the DNA replication phase. Two posttranscriptional events mostly account for this periodic fluctuation:

a) regulation of histone mRNA stability (see Eckner),

b) the regulation of the rate of 3' end formation of the histone mRNA (D. Schümperli, pers. comm.). This regulation also occurs during the transition from a quiescent to a proliferative state of the cell (see Hoffmann).

We have been studying the molecular mechanisms of histone RNA 3' end formation with the aim of understanding, at a molecular level, how the processing machinery recognizes

and cleaves the RNA precursor and to what extent these events are subject to modification in the cell cycle.

IN VITRO ASSAY. We constructed a minimal processing substrate by introducing a mouse H4 derived oligonucleotide downstream of an SP6 promoter. The RNA transcribed thereof comprises basically only the two processing signals flanking the cleavage site: a highly conserved hairpin structure and a purine-rich downstream spacer motif. When incubated in nuclear extracts from mouse hybridoma cells, this RNA undergoes an endonucleolytic cleavage producing the genuine mature 3' end. This demonstrates that the two conserved motifs are sufficient in vitro to direct fully efficient 3' processing of the histone precursor RNA.

HAIRPIN BINDING FACTOR (HBF). One obvious way to tune the efficiency of processing is to modulate the recognition of the precursor by the processing apparatus. Base-pairing between the downstream spacer element and the RNA moiety of the U7 snRNP has been shown to be essential for 3' end formation and this interaction appears to be downregulated in Go arrested cells (see Hoffmann). We were interested in defining the role of the other processing signal, a highly conserved stem-loop structure located upstream of the cleavage site. Mutational analysis showed that basepairing between the histone hairpin and the U7 snRNA is not required for efficient processing as previously proposed, but suggested that this motif acts as a target site for a distinct processing factor, the hairpin binding factor.

Competition experiments with various mutated RNA have allowed us to define the requirements for an efficient recognition of the histone element by a hairpin binding factor (HBF). The interaction between the inferred HBF and its target site requires not only the structural integrity but also the wild-type primary sequence of the stem-loop structure. Prevention of this interaction by mutation, linearization or complete deletion of the target site, or by addition of a suitable competitor RNA results in a loss of 80 - 90 % of the processing efficiency.

We further analyzed the behaviour of the HBF on various chromatography columns. The HBF distribution closely parallels that of the U7 snRNP although fractions can be obtained containing HBF activity but no detectable U7 snRNA. The tight association between the HBF and the U7 snRNA in buoyant density gradients further suggests that the HBF is,

under physiological conditions, an integral U7 snRNP protein. Fractions enriched in HBF activity were used for North Western blots analysis and revealed the presence of a 42 kDa protein which specifically reacts with the wild type RNA target site, but not with mutated palindrome sequences.

We are undertaking a large scale purification of the HBF and intend to purify it to homogeneity. Clearly, this necessary step will bring some light into the exact mechanisms of 3' processing and allow us to investigate whether this interaction is also subjected to regulation through the cell cycle.

HEAT LABILE FACTOR. The 3' processing reaction requires, in addition to the U7 snRNA and the HBF, the presence of a heat-labile factor. The heat-labile factor not only plays a crucial role in the 3' end formation of the histone mRNA, but also seems to be involved in their cell cycle regulation, as its activity fluctuates during the different stages of the cell cycle (see I. Hoffmann). In order to understand the exact role of the heat-labile factor, its purification has been undertaken.

Differential centrifugation proved to be an important first step, as the heat-labile factor could be purified and concentrated without major loss of activity. Further purification steps were carried out on a FPLC system, where different columns were tested for their applicability. The fractionation scheme included a cation exchange column, a hydroxyl apatite column and a molecular sieving column. Purified fractions analyzed by SDS-PAGE showed an enrichment of two sets of bands with an apparent molecular weight of 45 and 58 kDa. Further fractionation on strong anion exchanger and reactive dye columns removed contaminating snRNPs and provided fractions containing heat-labile factor but no detectable U7 RNA.

CsCl density gradients have also been used to purify the heat-labile factor further demonstrating that it can be dissociated from the U7 snRNA. The buoyant densities, however, suggest that the heat-labile factor may be associated with an RNA which, however, until now has evaded detection.

REGULATION OF U7-MEDIATED HISTONE PRE-mRNA 3' PROCESSING. *I. Hoffmann*

The 3' end formation of histone pre-mRNAs plays an important role in the cell cycle dependent regulation of histone genes. The expression of histone genes in proliferating

cells is tightly linked to the S phase of the cell cycle. The mechanisms of the histone pre-mRNA processing reaction in mammalian cells has been investigated. 3' end formation requires the U7 snRNP and at least two additional transacting processing factors, the hairpin binding factor and the heat-labile factor. The latter was shown to be limiting in Go arrested cells. Our interest is now focussed on the regulation of the 3' processing reaction during the cell cycle.

We observed that cells arrested in Go contain U7 snRNP particles whose RNA is completely protected against micrococcal nuclease attack whereas the U7 snRNP of exponentially growing cells, when subjected to micrococcal nuclease, loses 21 nt of its 5' terminus (the sequences interacting with the pre-mRNA). It is therefore suggested that a factor binds to the 5' terminal sequences of the U7 RNA in Go cells and prevents pre-mRNA/U7 snRNA interaction and as a consequence downregulates 3' processing, while in rapidly dividing cells the 5' end is capable of binding to the pre-mRNA and therefore permits 3' processing to occur. We have studied the transition from the proliferative to the non-dividing state of cells in the myoblast-myotubules cellular system (with Prof. B. Sells). We found that myotubules, whose 3' processing of histone mRNA is downregulated contain U7 snRNA which is fully protected against micrococcal nuclease and whose 5' end is apparently associated with the 5' binding factor.

Go cells can be brought into G1 and S phase after feeding them with medium with high serum content. It is observed that both the heat-labile factor and the release of the 5' binding factor are upregulated immediately prior to S phase, i.e., that the upregulation of 3' processing is a late regulatory event of the growth signal cascade which occurs after serum-stimulation of cells.

Serum-stimulation of cells arrested in the Go phase of the cell cycle shows that the regulation of both factors is coupled to DNA synthesis. Preliminary results indicate a 40 % higher molecular weight for the active U7 snRNP fractions in exponentially growing cells than for the inactive fractions of the cells arrested in the Go phase.

PURIFICATION OF U7 snRNPs AND CHARACTERIZATION OF THE PROTEIN MOIETY. *I. Hoffmann*

U7 snRNPs were fractionated from total nuclear extracts on DEAE sepharose, heparin sepharose and Mono Q columns. Active fractions were then further purified with a novel affinity chromatography method. This step is based on the hybridization of the free 5' end of U7 snRNA to a complementary DNA oligonucleotide which is biotinylated at the 3' end and bound via streptavidin to agarose at high salt concentrations. U7 snRNP containing fractions were eluted at low salt concentrations. Analysis of the fractions on a SDS protein gel reveals protein bands of 29kDa, 28kDa, 16kDa and 15kDa which correspond to the common snRNP proteins B, B', D, D', as could be shown by immunoblotting using antibodies against these proteins.

CELL CYCLE REGULATION OF HISTONE mRNA TURNOVER

R. Eckner and W. Ellmeier

The replication dependent histone transcripts are unique among vertebrate mRNAs in that they end at their 3' terminus with a stem-loop structure instead of the usual poly(A) tail. Histone transcripts are stabilized during S phase and destabilized during G1 phase of the cell cycle. The half-life of histone message is about 15 min. in G1 phase and 45 min. in S phase. This difference accounts for an 8 to 10-fold fluctuation in histone mRNA steady state levels between S and G1 phase. By treatment with inhibitors of protein synthesis (e.g. cycloheximide), histone transcripts can be superinduced in both phases of the cell cycle and they reach the same steady state level independent of the position of the treated cells in the cell cycle. These data suggested that ongoing translation is required for proper histone mRNA degradation. In addition, mutagenesis data, where the protein coding region has been altered in respect to its length and its position to the 3' terminal stem-loop structure, support the view that the actively translating ribosome recognizes an entity at the 3' end and only by doing so can it trigger a cell cycle-linked degradation signal. Furthermore, in vitro degradation assays suggest that a ribosome associated exonuclease degrades histone transcripts by introducing the first cuts at or nearby their 3' ends.

Proof that the histone 3' terminal stem-loop structure is required for the cell cycle dependent regulation of histone mRNA stability is still outstanding. One method of proving

the role of the stem-loop structure in the cell cycle would be to generate single base pair mutations in this structure which would completely abolish the regulation of histone mRNA degradation. Such an approach is however hampered by the multiple functions of the stem-loop structure during histone gene expression. In mammalian cells, this structure is an essential part of the 3' processing signal. By mutating it, one already interferes with histone gene expression at a very early step and never ends up with detectable mature histone mRNA. We have therefore devised a histone gene expression system that uses cis-acting ribozyme sequences to generate mature histone transcripts. By ribozyme mediated 3' end processing, it is possible to generate histone transcripts which expose mutated stem-loop structures at their 3' end. We have examined the efficiency of cleavage of such constructions in tissue culture cells (NIH 3T3 and HeLa cells) and we found that in all cases at least 90 % of the transcripts are properly cleaved. But in most cases a weak readthrough signal can be detected. We are currently testing the behavior of our collection of mutated transcripts regarding transport from the nucleus to the cytoplasm and in respect to their regulation of mRNA stability.

RIBOZYME, ANTISENSE RNA, AND ANTISENSE DNA INHIBITION OF U7 SMALL NUCLEAR RIBONUCLEOPROTEIN-MEDIATED HISTONE PRE-mRNA PROCESSING IN VITRO. *M. Cotten with G. Schaffner*

A comparative analysis of ribozyme, antisense RNA, and antisense DNA inhibitors of the in vitro (small nuclear ribonucleoprotein U7-dependent) histone pre-mRNA processing reaction was performed. Using the in vitro, histone pre-mRNA processing system described above (Vasserot & Tabiti), we compared the processing inhibition of antisense DNA and RNA and ribozyme molecules targeted to the U7 sequence. We found that antisense RNA was the most potent inhibitor of the processing reaction, blocking U7 activity (greater than 90 %) when present at a sixfold excess over the small nuclear RNA. Full-length (65 nucleotides [nt]) antisense DNA inhibited the reaction to the same extent at a 60-fold excess over U7, while a short (18-nt) antisense DNA molecule required a 600-fold excess to attain this level of inhibition. The inhibitory activity of the U7-targeted ribozyme was similar to that of the short antisense DNA molecule, requiring a 1,000-fold excess over the target RNA for complete inhibition. The DNA and ribozyme inhibitors however,

produced irreversible inhibition due to cleavage of the target RNA. Antisense RNA inhibition was reversible, presumably because of the dissociability of the RNA-RNA hybrid.

RIBOZYME MEDIATED DESTRUCTION OF RNA IN VIVO. *M. Cotten*

The targeted cleavage of RNA by ribozymes in trans had not as yet been observed in vivo. It has been shown, however, that the cleavage of U7 snRNA by U7 snRNA-targeted ribozymes requires a relatively high concentration of ribozymes to inhibit 3' processing of histone pre-mRNA in in vitro extracts, as compared for instance to complementary DNA or RNA oligomers, despite the catalytic properties of ribozymes (see above). The build-up of a concentration of ribozymes sufficient to elicit biological effects may in most instances become a limiting factor in vivo. If it were possible to introduce ribozyme synthesizing genes into the cell rather than the ribozyme itself, a considerable amplification would be achieved because such genes would produce a great many ribozymes and thus replenish the pools of ribozymes destroyed by nuclease activity.

We have chosen to test ribozyme tRNA genes in a Xenopus oocyte system. The large size of the oocyte nucleus allows one to introduce test genes and substrates by microinjection bypassing the variability of standard transfection into tissue culture systems, and eliminating the high background of untransfected cells obtained with most transient transfection methods. The large size of the cell facilitates the fractionation of nucleus and cytoplasmic material so that we can determine the cellular compartmentalization of gene products. Furthermore, the microinjection technique allows us to precisely control the amount of DNA and RNA that we are introducing into each cell. Frog oocytes have previously been used by others to great advantage for the study of antisense oligonucleotide dependent mRNA cleavage.

Transfer RNA genes, transcribed by pol III, are suitable "cassette" genes to express such ribozymes. Their small size (less than two hundred base pairs including the ribozyme coding sequence), their high rate of transcription and ubiquitous expression in different kinds of tissues make them good candidates for expressing ribozyme sequences. Earlier studies by others have demonstrated the utility of the pol III VAI promoter for the expression of antisense RNA.

We find that a very compact ribozyme producing gene unit can be constructed by simply placing the ribozyme coding sequences between the A and B block of a $tDNA^{met}$-gene. This gene, when injected into frog oocyte nuclei produces ribozymes in vivo. The ribozymes thus generated remain localized, in the main, inside the cell nucleus. However, sufficient ribtRNA migrated into the cytoplasm to cleave, and destroy, the U7 snRNA. Thus, the action of targeted "hammerhead" ribozymes in vivo is demonstrated.

IN VIVO APPLICATION OF RIBOZYMES. *H. Kandolf*

The ability of tRNA-ribozymes (tRNArib) to cleave mRNAs at specific sites in vitro and in vivo has been demonstrated (see above report). The construct used, consisting of a ribozyme cloned into the anticodon stem-loop region of a $tDNA^{met}$ gene, is functional in Xenopus laevis oocytes, but not with an activity as high as desired. The resulting transcript has apparently lost its ability to form a tRNA-like secondary structure. Hence the construct remains in its precursor form, mainly in the nucleus. The stability does not seem to be increased compared with unprotected ribozymes used in former in vitro assays, where a 1000-fold excess over a substrate RNA was needed for complete cleavage.

For this reason, investigations were initiated in order to increase the efficacy of the tRNArib system. Initial experiments showed that extension of the anticodon stem restores the transcripts' ability to form the tRNA secondary structure so that processing can occur. This leads to a 6-fold higher level of tRNArib molecules in Xenopus oocytes compared to a poorly processed construct containing a truncated anticodon stem.

Moreover, we are beginning to investigate the significance of histone H1 variants. We will use 2'-methoxy antisense oligoribonucleotides and ribozymes to specifically inhibit H1 expression during early developmental stages of Xenopus laevis. We hope to achieve efficient repression of single H1 histone variants which would enable us to study possible biological effects due to the disappearance of a H1 subtype.

TRANSFERRIN-POLYCATION CONJUGATES AS CARRIERS FOR DNA UPTAKE INTO CELLS. *M. Cotten with M. Zenke, Ernst Wagner and H. Beug*

We have developed a high efficiency nucleic acid delivery system which uses receptor-mediated endocytosis to carry DNA macromolecules into cells. We have accomplished

this by modifying the iron transport protein transferrin by the addition of polycationic nucleic acid binding domains. Human transferrin as well as the fowl homologue conalbumin have been covalently linked to the small DNA binding protein protamine or to polylysines of various sizes, using a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor, are rapidly endocytosed and maintain their capacity to bind and deliver iron to the cell. The polycation-transferrin molecules form electrophoretically-stable complexes with double-stranded DNA, single-stranded DNA and modified RNA molecules independent of the size of the nucleic acid (ranging from short oligonucleotides to DNA of 21 kilobasepairs). When complexes of polycation-transferrin and a bacterial plasmid DNA containing the gene for Photinus pyralis luciferase are supplied to eukaryotic cells, high level expression of the luciferase gene occurs, demonstrating transferrin receptor mediated endocytosis and expression of the imported DNA. We refer to this delivery system as transferrinfection.

THE EFFICIENCY TRANSFERRINFECTION CORRELATES WITH TRANSFERRIN RECEPTOR NUMBER ON THE CELL SURFACE. *M. Cotten with Ernst Wagner, M. Zenke, H. Beug, F. Längle-Rouault, Helen Kirlappos*

The higher eukaryotic cell senses its iron levels as a function of the internal iron-heme concentration. Compounds which alter the internal iron levels or modify the level of heme, either by decreasing heme synthesis or increasing heme degradation, result in a modulation of transferrin receptor levels. If, for instance, one chelates the internal iron pool with the cell permeable iron chelator desferrioxamine, the cell responds to the induced iron deficit by increasing the number of transferrin receptors on its surface. Conversely, if a cell is supplied with soluble iron salts, it responds to the resulting iron glut by lowering its transferrin receptor levels. The compound succinylacetone is a potent inhibitor of the first committed step of heme biosynthesis, 5-aminolevulinic acid dehydratase. Treatment of HeLa cells with this compound results in an approximate 2-fold increase in cell-surface transferrin receptors. A similar observation has been made with cultured rat hepatocytes. If one alters the heme content of a cell from the opposite direction, that is, by stimulating heme degradation by increasing the heme oxygenase activity of the cell, the cell also responds by increasing the transferrin receptor levels. This has been demonstrated in

HeLa cells; treatment of the cells with the heme oxygenase stimulator cobalt chloride (and to a lesser extent tin chloride) results in an increase in transferrin receptor levels. If one directly chelates the internal iron pool with the cell-permeable iron chelator desferrioxamine, the cell also responds to this iron deficit by increasing the number of transferrin receptors on its surface.

We have tested the effects of pretreating cells with the heme oxygenase stimulator, $CoCl_2$, with the intracellular iron chelator, desferrioxamine, and with the 5-aminolevulate dehydratase inhibitor, succinylacetone pretreatment on gene expression following transferrinfection with polylysine-transferrin. We find that in K562 cells, which express functional transferrin receptors, pretreatment with desferrioxamine results in a 10-fold increase in gene expression. Pretreating K562 cells with cobalt chlorides results in a similar stimulation of gene expression; the combination of both desferrioxamine and cobalt results in a further stimulation of expression over the level obtained with either compound alone, consistent with the compounds interfering with distinct metabolic steps. Succinylacetone stimulates expression to a lesser extent than desferrioxamine, but once again, a combination of the two compounds results in higher expression than with either compound alone.

LIST OF PUBLICATIONS

Birnstiel, M.L., Schaufele, F. (1988) in Birnstiel, M.L., (ed.),
Structure and Function of Major and Minor snRNAs. Springer Verlag, Berlin.

Cotten, M., Gick, O., Vasserot, A., Schaffner, G., Birnstiel, M.L. (1988). Specific contracts between mammalian U7 snRNA and histone precursor RNA are indispensable for the in vitro 3' RNA processing reaction. EMBO J. 7, 801-808.

Cotten, M., Schaffner, G. and Birnstiel, M.L. (1989). Ribozyme, Antisense RNA and Antisense DNA inhibition of U7 snRNP mediated histone pre-mRNA Processing in Vitro. Mol. Cell Biol. 9, 4479-4487.

Vasserot, A.P., Schaufele, F. and Birnstiel, M.L. (1989). Conserved terminal hairpin sequences of histone mRNA precursors are not involved in duplex formation with the U7 RNA but act as a target site for a processing factor. Proc. Natl. Acad. Sci. USA 86 4345-4349.

Eckner, R. and Birnstiel, M.L. (1989). Cloning of cDNAs coding for human HMG I and HMG Y proteins: both are capable of binding to the octamer sequence motif. NAR 17, 5947-5958.

Cotten, M. and Birnstiel, M.L. (1989). Ribozyme mediated Cleavage of RNA in vivo. EMBO J. 8, 3861-3866.

Birnstiel, M.L. and Busslinger, M. (1989). Dangerous Liaisons: Spermatozoa as Natural Vectors for Foreign DNA? Cell 57, 701-702.

Wagner, E., Zenke, M., Cotten, M., Beug, H. and Birnstiel, M. (1990). Transferrin-polycation conjugates as carriers for DNA uptake into cells. PNAS, in press.

Cotten, M., Längle-Rouault, F., Wagner, E., Zenke, M., Beug, H., Kirlappos, H. and Birnstiel, M. (1990). Stimulation of polylysine-transferrin mediated DNA transferrinfection by agents which affect the survival of transfected DNA or modulate transferrin receptor levels. PNAS, in press.

Meinrad BUSSLINGER	**Senior Scientist**
Benjamin ADAMS	**Postdoc**
Luigi VITELLI	**Postdoc**
Katharina WIDENHORN	**Postdoc**
Christopher WRIGHTON	**Postdoc**
Giulio SUPERTI-FURGA	**PhD Student**
Sylvia BRASELMANN	**PhD Student**
Gabriele BERGERS	**PhD Student**
Petra DÖRFLER	**PhD Student**
Paula GRANINGER	**Technician**

Transcription factors involved in signal transduction and differentiation

A) The role of the oncoprotein *Fos* in signal transduction

Introduction

Tumour cells differ from normal cells in many different ways that relate mainly to altered control of cell proliferation or differentiation. The abnormal phenotype of tumour cells is caused by one or several activated oncogenes resulting in reprogramming of gene expression. The products of these oncogenes often act at different points in the signal transduction pathway that is normally responsible for the proper phenotypic response of a cell to changes in its environment. The proto-oncogene *c-fos* is one of the prime targets of the signal transduction pathway in the nucleus. The *Fos* protein is known to be the most important component of the transcription factor complex AP1 which is thought to act as a master regulator by coupling

TRANSKRIPTIONSFAKTOREN
WELCHE ANDEN SIGNALÜBERTRAGUNG
UND DIFFERENZIERUNG BETEILIGT
SIND

short-term stimulation of a cell to long-term alteration of gene expression (reviewed by Curran, 1988). The aim of our research programme is to study the biological role of *Fos* by identifying the genes that are regulated by this protein.

Fos-regulated genes can in principle be identified in two different ways. In the so-called *"loss of function"* experiment the expression of the endogenous *fos* gene is interfered with at the post-transcriptional level. *Fos*-responsive genes should consequently fail to respond to stimulation by the signal transduction pathway. Inhibition of *fos* expression can be achieved either by blocking *fos* mRNA with anti-sense transcripts, by destroying *fos* mRNA with specific ribozymes or by over-expressing trans-dominant mutant *Fos* proteins that interfere with AP1 function (see report of Martin Nicklin). In the *"gain of function"* experiment an exogenous *fos* gene under the control of an inducible promoter is introduced into the cell. Stimulation of exogenous *Fos* synthesis should result in transcriptional activation or repression of *Fos*-responsive genes. Both approaches will allow the isolation of *Fos*-regulated genes by differential cDNA cloning techniques (Duguid et al., 1988).

In the last year we have started to apply above strategies to two different cellular systems; rodent fibroblasts (NIH 3T3, 208F, Rat 1a) and rat PC12 cells. A link between proliferation control, transformation and *Fos* gene expression has been firmly established in the fibroblast system (reviewed by Verma, 1986). Our experiments are designed to elucidate the molecular nature of this link. The rat phaeochromocytoma cell line PC12 can be differentiated with nerve growth factor (NGF) into sympathetic neuron-like cells. *Fos* gene expression is thought to be important at the onset of this differentiation programme (Milbrandt, 1986). PC12 cells may therefore provide insight into the role of *Fos* in differentiation.

Characterization of a selective Fos induction system

Sylvia Braselmann, Paula Graninger

We have developed and characterized a novel induction system for conditional expression of an exogenous fos gene, which is based on the following two ideas. First, the cell lines of

interest (NIH 3T3, 208F, Rat 1a, PC12) do not contain any endogenous oestrogen receptor level. Hence, oestrogen is an inert signal for these cells. Second, there is no evidence to date for the existence of a mammalian homologue of the yeast Gal4 protein. The probability is therefore low that multiple Gal4 binding sites exist near genes in the mammalian genome. For these reasons a hybrid transcription factor consisting of the DNA binding domain of the yeast Gal4 protein and of the hormone-binding domain of the human oestrogen receptor (Webster et al., 1988) should trans-activate only a synthetic Gal4-responsive promoter in transfected cells (see Figure 1). Our most optimal promoter construct consists of four Gal4-binding sites upstream of a CCAAT element and a TATA-box. This promoter is 30-50-fold inducible by oestrogen in cell lines that constitutively express the Gal-ER protein. The induced level of activity of this promoter is as high as that of the SV40 enhancer/promoter in fibroblasts. Using this induction system we have established stable NIH 3T3 transformants containing the following three genes: a constitutively expressed Gal-ER gene, a *c-fos* gene under the control of the Gal4-responsive promoter and

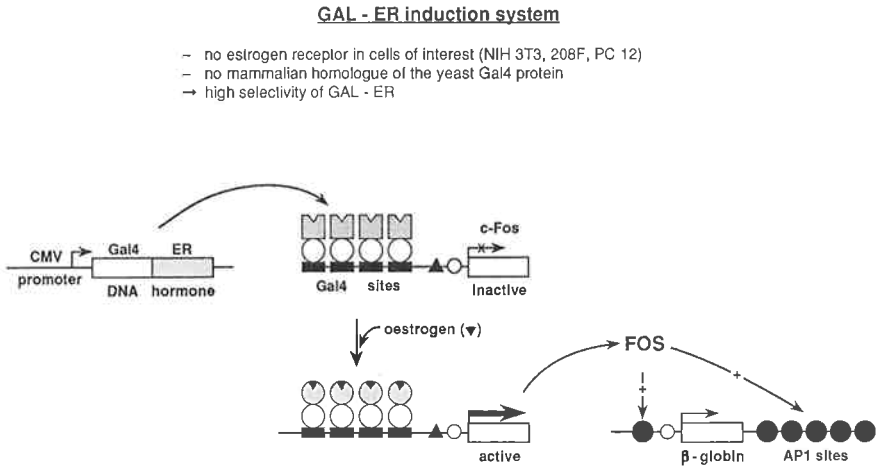


Fig. 1 Gal-ER induction system for conditional *Fos* expression.

a *Fos*-responsive β-globin gene containing an AP1 site as an upstream promoter element and five AP1 sites as a downstream enhancer (Figure 1). Two cell lines were characterized that show clear oestrogen-dependent *fos* expression. *Fos* synthesis in turn results in increased

activity of the β-globin gene indicating that the regulatory cascade shown in Figure 1 is fully functional in these cell lines. We are currently studying the kinetics of known endogenous *Fos*-responsive genes in this system and are analyzing oestrogen-dependent transformation of these cell lines.

The role of Fos for PC 12 cell differentiation *Christopher Wrighton*

The PC12 cell line has been extensively used to study the process of NGF-mediated neuronal differentiation. NGF treatment triggers the cycloheximide-insensitive expression of a number of immediate early genes including the *c-fos* gene as part of a highly complex reprogramming of gene activity. Beginning with the *fos* gene, it is our objective to start to dissect these pathways using a highly selective induction system to drive the expression of individual immediate early genes in PC12 transfectants. Established systems based on glucocorticoid induction cannot be used in this case since these agents appear to antagonized the effects of NGF. We have instead established stable PC12 cell lines that constitutively express a transfected gene encoding the oestrogen-regulable Gal-ER transcription factor. We are presently introducing *fos*, antisense-*fos* and trans-dominant mutant *fos* genes under the control of a synthetic Gal4 promoter into these cell lines so as to define the role of *Fos* in the differentiation process and ultimately to identify PC12 cell-specific *Fos*-responsive genes by differential cDNA cloning.

Hormone-dependent activity of Fos-steroid receptor fusion proteins
Giulio Superti-Furga, Gabriele Bergers

The hormone-binding domain of the oestrogen and glucocorticoid receptor contains an inactivation function that renders the activity of a fusion protein ligand-dependent. The trans-activation function of the adenovirus E1A protein and the transforming activity of the *Myc* protein have been successfully put under hormonal control in this way (Picard et al., 1988; Eiler et al., 1989). We have constructed chimaeras between the mouse *Fos* protein and the

C-terminal region of the human oestrogen or the rat glucocorticoid receptor. Transient transfection experiments revealed that these hybrid proteins are able to trans-activate a test gene containing AP1 binding sites in a hormone-dependent manner in several different cell lines (NIH 3T3, HeLa, CV-1 and F9). Moreover, stable integration of a gene coding for the hybrid *Fos-ER* protein results in reversible oestrogen-dependent transformation of rat fibroblasts. A clear advantage of this induction system is its short lag time as the pre-synthesized inactive form of the *Fos-ER* protein can immediately be activated by hormone addition. Ultimately we will use these cell lines to identify the genes involved in Fos-mediated transformation of fibroblasts.

B) Characterization of a novel B-cell-specific transcription factor

Introduction

Previous work of our lab led to the identification of a novel transcription factor that interacts with the promoter of four tissue-specific late histone H2A-2 and H2B-2 genes of the sea urchin. The binding site for this factor was shown to be absolutely required for efficient transcription of these genes. Moreover, the abundance of this protein during early development and in adult tissues correlates well with the expression pattern of the late H2A-2 and H2B-2 genes. We therefore refer to this transcription factor as tissue-specific activator protein (TSAP). TSAP may be a master regulator of a family of genes that are all expressed in the same cell-type-specifc manner as the late H2A-2 and H2B-2 genes during sea urchin ontogeny. Transcription factors are often highly conserved during evolution. For this reason we have chosen to search for the homologous protein in the experimentally more amenable mammalian system rather than to further characterize the sea urchin TSAP protein.

BSAP: A novel transcription factor present at early but not late stages of B-cell differentiation *Katharina Widenhorn, Luigi Vitelli, Ben Adams, Petra Dörfler*

A novel B-cell-specific transcription factor, BSAP, was identified as a mammalian homologue of the sea urchin TSAP by the following criteria. First, the mammalian protein binds specifically and with equal affinity to the TSAP binding sites of all four sea urchin H2A-2 and H2B-2 genes. Second, as shown by methylation interference experiments, this protein contacts the same bases of the recognition sequences as the sea urchin TSAP. Third, a single point mutation at the only strictly invariant position within the TSAP recognition sequences drastically reduces DNA binding of both the sea urchin TSAP and the mammalian BSAP protein. Screening a variety of different cell lines revealed that BSAP is exclusively present in B-lymphoid cells. Moreover, this protein is present at similar concentrations from an early progenitor B-cell up to the mature B-cell stage. BSAP is, however, absent in terminally differentiated, immunoglobulin-secreting plasma cells. BSAP was furthermore shown to be a transcription factor, as a wild type, but not a mutant TSAP-binding site of the sea urchin functions as an upstream promoter element in transfected B-cells, but not in plasma, HeLa or T-cells. Hence we refer to this protein as B-cell-specific activator protein (BSAP). Competition experiments did not reveal any high affinity binding site for BSAP in known regulatory regions of immunoglobulin and class II MHC genes, suggesting that BSAP is a regulator of a different set of B-lymphoid-specific genes. Indeed, the expression pattern of three genes coding for the B-cell-specific differentiation antigenes mb-1, Lyb-2 and CD19 correlates well with that of BSAP suggesting that BSAP may control these genes. As a next step towards understanding the role of BSAP in B-cell differentiation we are now cloning the gene coding for this transcription factor.

Publications during the year:

Superti-Furga, G., Barberis, A., Schreiber, E., and Busslinger, M. (1989). The protein CDP, but not CP1, footprints on the CCAAT region of the g-globin gene in unfractionated B-cell extracts. Biochim. Biophys. Acta 11007, 237-242.

Superti-Furga, G., and Busslinger, M. (1989). Nuclear factors interacting with the promoter of human g-globin genes. Highlights Modern Biochem. Vol. 1, 531-540 (A. Kotyk et al., eds; VSP Utrecht, The Netherlands)

Southgate, C., and Busslinger, M. (1989). *In vivo* and *in vitro* expression of U7 snRNA genes: cis- and trans-acting elements required for RNA polymerase II-directed transcription. EMBO J. 8, 539-549.

Birnstiel, M., and Busslinger, M. (1989). Dangerous liaisons: Spermatozoa as natural vectors for foreign DNA? Cell 57, 701-702.

Barberis, A., Superti-Furga, G., and Busslinger, M. (1989). The testis-specific octamer-binding protein of the sea urchin has a molecular weight of 85 kDa. Nucleic Acids Res. 17, 7114.

Barberis, A., Superti-Furga, G., Vitelli, L., Kemler, I., and Busslinger, M. (1989). Developmental and tissue-specific regulation of a novel transcription factor of the sea urchin. Genes Dev. 3, 663-675.

Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1989). A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. Genes Dev., submitted.

Other references:

Curran, T. (1988) The *fos* oncogene. in *The oncogene handbook*. E.P. Reddy, A.M. Skalka and T. Curran, eds. (Elsevier, Amsterdam), pp. 307-325.

Duguid, J.R., Rohwer, R.G., and Seed, B. (1988) Proc. Natl. Acad. Sci. USA 85, 5738-5742.

Eiler, M., Picard, D., Yamamoto, K.R., and Bishop, M.J. (1989) Nature 340, 66-68.

Milbrandt, J. (1986) Proc. Natl. Acad. Sci. USA83, 4780-4793.

Picard, D., Salser, S.J., and Yamamoto, K.R. (1988) Cell 54, 1073-1080.

Verma, I.M. (1986) Trend Genet., 93-96.

Webster, N.J.G., Green, S., Jin, J.R., and Chambon, P. (1988) Cell 54, 199-207.

Kim NASMYTH	**Senior Scientist**
D. LYDALL	**Postdoc**
I. MUROFF	**Postdoc**
Uttam SURANA	**Postdoc**
Graham TEBB	**Postdoc**
Angelika AMON	**PhD Student**
Thomas MOLL	**PhD Student**
Shantini SOCKANATHAN	**PhD Student**
M. Rita TABA	**PhD Student**
Helmut ROBITSCH	**Technician**

GENETICS OF YEAST CELL CYCLE CONTROL AND DIFFERENTIATION

Cell cycle control

During the last two years, we have witnessed a major development in our understanding of how the cell cycle is regulated. Until recently, there were two somewhat independent foci of cell cycle research: studies on the regulation of maturation promoting factor (M.P.F.) in Xenopus or marine invertebrate eggs and the quite separate genetic studies of cell cycle control in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The former had suggested that M.P.F. was not just an oddity concerning oocyte maturation but was a factor which, through oscillations of its activity, also drive the mitotic cell cycles of cleaving embryos. The yeast studies, on the other hand, had identified two genes, *CDC28* from *cerevisiae* and *cdc2* from *pombe*, which encode homologous protein kinases which are necessary for the initiation of both DNA replication and mitosis in these organisms. The recent critical breakthrough has been the discovery that the vertebrate homologue of the CDC28/cdc2 kinase is a vital component of M.P.F. and is synonymous with the previously

known cell cycle dependent histone H1 kinase. It is now proposed that M.P.F. is no more than an active form of the CDC28/cdc2 kinase and that oscillations in its activity are responsible for the timing of mitosis in all eukaryotic organisms. Increases in activity cause entry into metaphase whereas destruction of kinase cause the metaphase to anaphase transition. It has also been discovered that a protein called cyclin (also conserved in yeast) is an important co-factor for the kinase and that oscillations in the levels of cyclin during the cell cycle are involved in determining the pattern of kinase activity.

Given this framework, one may pose at least two sorts of questions concerning cell cycle control. First, how is the activity of the CDC28/cdc2 kinase itself regulated? How exactly does the kinase activity vary during the cell cycle? In *Saccharomyces cerevisiae*, preliminary measurements (see under **control of CDC28 kinase activity**) suggest that there is little or no Histone HI Kinase activity in early G1 cells, that an increase in activity occurs as cells undergo START, i.e commit themselves to DNA replication, that a further increase occurs somewhat later in the cell cycle and may drive cells into a metaphase-like state (i.e form a mitotic spindle), that a reduction of kinase may signal the initiation of anaphase, and finally that the activity disappears when cells enter G1, thus completing the cycle. What determines these changes? Second, to what extent do such variations in the CDC28/cdc2 kinase activity drive significant events of the cell cycle and how does the kinase exert its effect? That is, we need to identify the substrates of the kinase and to determine how their biological activity is altered by CDC28 phosphorylation. Much of the work in the yeast lab is directly concerned with these sorts of questions. We are studying how the *HO* and *SWI5* genes are only activated at certain stages of the cell cycle (possibly triggered by changes in CDC28 kinase activity), how the CDC28 kinase is itself regulated during the cell cycle, how pheromones regulate the cell cycle, and how the localization of the SWI5 transcription factor may be directly regulated by its state of phosphorylation by the CDC28 kinase.

Origin of cell differentiation

One of the outstanding questions in developmental biology is how different patterns of gene expression arise within a clone of cells with identical genotype. One of the simplest examples of this phenomenon occurs when a haploid homothallic yeast cell self-diploidizes through mating type switching.

Saccharomyces cerevisiae has two mating types which are determined by the the **a** and alpha alleles of the *MAT* locus. In heterothallic (*ho*) strains, these mating types are stable. Cells descended from germinating haploid spores remain haploid unless mixed with cells of opposite mating type. In contrast, haploid spores derived from homothallic (*HO*) strains, which are born with either an **a** or an alpha mating type, rapidly become diploid without encountering cells of differing clonal origin because they rapidly switch their mating types and produce diploids through the mating of clonal cousins. Homothallic cells switch their mating types in a very specific pattern. Germinating spores undergo cell division to produce a mother cell which proceeds to switch its mating type during its next cell division and a daughter cell which does not. We now know that the pattern of mother-specific mating type switching is determined by the pattern of transcription of the HO endonuclease gene which initiates mating type switching by making a double stranded break in the *MAT* locus. *HO* is transiently transcribed when mother cells undergo START during G1 (i.e upon commitment to the mitotic cell cycle) but not at the equivalent period of the daughter cell cycle.

Our goal is to understand why *HO* transcription is confined to mother cells. We have identified what we believe to be the crucial transcription factor which is missing in daughter cells as they undergo START. This is a zinc finger site-specific DNA binding protein encoded by the *SWI5* gene. We are now trying to understand how this protein achieves asymmetric activation.

Control of CDC28 kinase activity

U. Surana, H. Robitsch

Genetic evidence suggests that the protein kinase encoded by the *CDC28* gene is a key regulator of the cell cycle in the budding yeast *Saccharomyces cerevisiae*. The kinase is highly conserved in all eukaryotic organisms and in the case of the fission yeast *Schizosaccharomyces pombe* is encoded by the *cdc2* gene. In fission yeast and in mammalian cells, the cdc2 kinase plays a vital role in driving cells into mitosis. In budding yeast, on the other hand, it has been suggested that CDC28 is unnecessary for mitosis and is only required for the initiation of S phase, a role which it also plays in fission yeast. In order to clarify the role of CDC28, we have set out to measure, using techniques different to those used previously, when exactly the kinase is active during the cell cycle. We find that there is little or no activity in pre-START G1 cells, that the kinase becomes active at the beginning of S phase, that it increases in activity as cells enter mitosis, and finally that there is a significant level of kinase until the very end of mitosis. We suspect, therefore, that the kinase does play an important role in mitosis even in budding yeast.

During the course of this work, we have characterized an unique allele of the *CDC28* gene (*cdc28-1N*) which, unlike all other alleles of *cdc28*, causes cells to arrest in a metaphase-like state at the restrictive temperature. We have discovered that, surprisingly, this allele is not defective in kinase activity and have therefore proposed that the mutant cells become arrested in metaphase because their kinase does not signal its own destruction, an event which is thought to be necessary for the metaphase to anaphase transition. If this hypothesis is correct, then it is clear that further chararcterization of the mutant promises to provide fundamental insights into how cells effect this critical stage of mitosis.

Identification of essential genes in the pheromonal signal transduction pathway

A. Amon

This work started off with a question concerning cell cycle control but has since drifted into the area of pheromonal signal transduction. We were interested in understanding how the yeast pheromones **a** and alpha factor arrest cells in the G1 phase of the cell cycle. In *S. cerevisiae*, the best way of identifying genes involved in this process is to look for mutants which fail to arrest in the presence of pheromone. To this end, we have developed a screen for mutants with an **a** mating type which would express the *HO* gene in the presence of alpha factor. Since *HO* is not expressed in G1 but is activated soon after START, we have an indicator of whether cells have initiated a new cell cycle. The screen has the advantage that it would be possible to identify essential genes. In order to avoid merely isolating mutants defective in signal transduction, we demanded of the mutants that they still be capable of expressing the alpha factor inducible *FUS1* gene. Out of 45 mutants, we have chosen to study 8 which also caused ts lethality. These 8 mutants defined 4 complementation groups called *arl*1, 2, 3, and 4 (*arl* stands for alpha factor resistant lethal). Surprisingly, despite our demand that all mutants be capable of inducing the *FUS1* gene, all four *ARL* genes are, after all, involved in signal transduction. Upon being shifted to the restrictive temperature, the mutants respond more or less normally to alpha factor during the first hour, i.e *FUS1* is induced, but the cells then rapidly lose the ability to induce *FUS1* and simultaneously start to bud. The mutants fail to enter a second cell cycle at the restrictive temperature.

In order to identify a step in the signal transduction pathway at which *arl* mutants are defective, we took advantage of a recently isolated mutation *GPA1-1* in the α subunit of the G-protein which causes constitutive activation of the signal transduction pathway. Northern analysis of *FUS1* mRNA of *GPA1-1*, *arl* double mutants showed the same pattern of *FUS1* induction as *arl* 1 to 4 single mutants did. From these data we conclude that the *arl* gene products act at a later step of the signal transduction pathway than the α subunit. *Arl* defects were observed in cells of both mating types, which shows that they are not mating-type-specific.

Since *arl1* mutants had the most homogeneous phenotype in the presence of alpha factor (95% of the cells ended up "shmooed" with a bud on the tip), we decided to analyse this complementation group in more detail. The gene was cloned and sequenced but did not show any significant homology to any DNA or protein found in the data base. *ARL1* has an open reading frame of 1.7 kb and encodes a protein of 568 amino acids. We have also obtained suppressors of *arl1-1*; all of them are dominant and show a temperature dependent resistance to alpha factor. They are sensitive to the pheromone at the permissive temperature but are resistant to it at the restrictive temperature. This indicates that the suppressors rescue the lethality of *arl1* but are not able to compensate its defect within the signal transduction pathway, since the suppressor mutation alone behaves like wild type cells in the presence of alpha factor.

It seems, therefore, that the *ARL* genes are not specifically altered in alpha factor cell cycle control but are, rather, essential genes involved in the signal transduction pathway.

Cell cycle regulated *SWI5* transcription

D. Lydall

SWI5 encodes a transcription factor essential for the expression of the *HO* gene. Considerable evidence (see under **a determinant of mother cell specific *HO* expression**) suggests that SWI5 is the major determinant of mother cell specific *HO* activation; that is, it is the only factor missing when daughter cells undergo START. A property of the *SWI5* gene vital for this role is its repression during the G1 phase of the cell cycle. The *SWI5* gene is inactive throughout G1, is only activated at some point during S phase, and remains active up to the end of mitosis. This regulation is necessary to prevent *HO* being expressed in daughter cells. We would like to understand how the *SWI5* gene is inactivated as cells complete mitosis and enter G1 and how it is re-activated as cells enter S phase. To this end, we have characterized the *SWI5* promoter by deletion analysis and have

set up a genetic screen which has enabled us to isolate mutants which are altered in *SWI5* regulation. We have identified a 50 bp DNA sequence from the *SWI5* promoter which is sufficient to confer G1-specific repression on a *lacZ* reporter gene and have also discovered a protein in crude yeast extracts which binds to this sequence. We are currently characterizing mutants which either fail to turn the gene on or activate it constitutivley during the cell cycle.

A determinant of mother cell specific *HO* gene expression

K. Nasmyth, T. Moll, G. Tebb

Genetic studies have implicated the *SWI5* gene in the asymmetric transcription of the *HO* endonuclease gene; that is, in confining the expression of *HO* to Mother cells. The simplest hypothesis to explain how this asymmetry is accomplished postulates that the SWI5 protein is itself differentially segregated at mitosis so that Daughter cells are "born" without any SWI5. Since transcription of SWI5 is known not to be initiated until after the window of possible *HO* transcription (see under **cell cycle regulated *SWI5* transcription**), this mechanism would be sufficient to account for the observed asymmetry.

The hypothesis has been tested by using antibodies to visualize the intracellular location of the SWI5 protein by means of indirect *in situ* immunofluorescence. The results of such studies have shown that:

a) the location of SWI5 is cell-cycle regulated. The protein is confined to the cytoplasm from S-phase, when transcription of the SWI5 gene is initiated, until some point in late anaphase when it enters the nucleus and is concentrated there (see under **cell cycle regulated nuclear localization**). The nuclear form of SWI5 appears to be unstable and, as a result, G1 cells contain barely detectable levels of the protein; and

b) Mother and Daughter cells appear to inherit SWI5 equally. When SWI5 moves from the cytoplasm to the nucleus at the end of anaphase, it seems to be concentrated equally in the mother and daughter nuclei.

Since there is apparently no significant asymmetry in the distribution of SWI5 at cell division, other hypotheses for asymmetry must now be entertained. According to the "timing" hypothesis, *HO* is activated only in mother cells by virtue of their shorter G1 period. The SWI5 protein which enters the nucleus of both mother and daughter cells is unstable as measured by the cell cycle dependent disappearance of immuno-reactive material on Western blots and by the disappearance of nuclear accumulated protein as seen by *in situ* immunofluorescence as cells proceed from late anaphase into the next G1 period. This suggests that asymmetry in *HO* expression could, in principle, arise from the known difference of G1 length in mother and daughter cells. Daughter cells are usually smaller than mother cells at birth and as a consequence of a requirement for a critical cell mass before cells can undergo START, have to spend significantly longer than mother cells in G1 prior to the START dependent activation of *HO*. This suggests that the SWI5 protein inherited by daughter cells may disappear before these cells have a chance (which is confined to a window in the cell cycle at or soon after START) of turning on the *HO* gene. Indeed, the observation that constitutive expression of *SWI5* throughout G1 is sufficient to activate *HO* in daughter cells implies that the SWI5 normally inherited by daughter cells at anaphase must decay to insignificant levels by the time they undergo START.

Important data relating to this hypothesis have come from analysing a series of in-frame deletions within the *SWI5* gene. Suprisingly, much of the SWI5 protein can be deleted without compromising its ability to activate *HO*. As a consequence, it has been possible to test whether the protein contains specific regions necessary for its mother cell specific activation of *HO*. Deleted versions of the gene were transfered to the *SWI5* locus and the pattern of mating type switching was observed. This showed that a specific region in the middle of the SWI5 protein is necessary for preventing it from activating *HO* in daughter cells. The observation that daughter nuclei actually inherit SWI5 protein led us to postulate that this region of SWI5 causes the protein to be so unstable that insufficient amounts of the protein inherited by daughter cells survives until *HO* can be activated at START. In order to test this hypothesis, we have analysed the levels of *SWI5* mRNA and protein from the wild type and mutant genes as cells complete mitosis and enter G1. We find that whereas both

the mRNA and protein from the wild type gene are very rapidly degraded as cells enter G1, the protein encoded by a deleted form of the gene which causes daughter cell switching is only very slowly degraded with the result that a significant amount is left as the cells undergo START. We believe, therefore, that rapid degradation of the SWI5 protein inherited by daughter cells is essential to prevent them from expressing *HO*.

The phenotype of *SWI5* deletion mutants is therefore consistent with the timing hypothesis, but the data do not go so far as to prove that it is correct. The SWI5 protein which enters mother nuclei is also very unstable and most of it has probably disappeared by the time that they initiate *HO* transcription. The critical question regarding mother/daughter asymmetric *HO* expression now concerns how mother cells manage to have sufficient SWI5 when they undergo START. The timing hypothesis proposes, of course, that their G1 period is sufficiently short for there still to be enough SWI5 remaining by the time that cells undergo START. In order to test this idea further, we are trying to analyse the consequences of lengthening the pre-START G1 period of mother cells.

Should the timing hypothesis prove to be incorrect, it will become likely that the SWI5 content of Mother cells is somehow different from that of Daughter cells. This could be because some of the SWI5 of Mother cells is present in the form of a stable complex on the *HO* promoter which is resistant to degradation by the normal mechanism. To investigate this possibility the binding sites for SWI5 within the *HO* promoter have been identified. Using antibodies it has been demonstrated that SWI5 binds *in vitro* to two sites within the proximal 1950bp of the promoter. These sites have been further defined by methylation interference and are currently being mutated with a view to introducing the mutations into the genome and examining the effect on *HO* transcription. It is also hoped to develop techniques to assay the binding of SWI5 to the *HO* promoter *in vivo*.

Cell cycle regulation of nuclear localization

T. Moll, G. Tebb, U. Surana, K. Nasmyth

During the course of analysing the distribution of the SWI5 protein at cell division with the aim of addressing the mechanism of mother/daughter specificity, we discovered that the SWI5 protein varies in its cellular location during the cell cycle. During the S, G2, and M phases, the protein accumulates in the cytoplasm but then, very suddenly, enters the nucleus as cells enter G1. We have now analysed the sequences within SWI5 required for its nuclear localization and discovered that a crucial determinant is a highly positively charged region at the C-terminus of the protein. Besides its basic nature (a property in common with most other nuclear import signals), we have noticed that the putative nuclear localization sequence of SWI5 contains two to three sequences which match the consensus sequence for the growth regulated histone H1 protein kinase, i.e the CDC28/cdc2 kinase. This has led us to propose that the cell cycle regulated nuclear entry of SWI5 may be due to cell cycle regulated phoshorylation by the CDC28 protein kinase. More specifically, we propose that phosphorylation by CDC28 inhibits the SWI5 nuclear localization signal. So far, several important predictions of this hypothesis have been fulfilled:

1) purified SWI5 protein is an efficient substrate for the CDC28 kinase in vitro.

2) Using different *cdc* mutants which arrest at different stages of the cell cycle, that there is a strong correlation between the existence of SWI5 in the cytoplasm and the level of CDC28 kinase activity. The protein only accumulates in the nucleus when there is little or no CDC28 kinase activity in the cell.

3) SWI5 enters the nucleus as cells finish mitosis and enter G1. We have carefully measured the CDC28 kinase during this period of the cell cycle and have found that there is a dramatic drop in kinase activity at precisely this stage in the cell cycle.

4) We have shown that SWI5 is a phosphoprotein and that the phosphorylation of certain tryptic peptides is cell cycle relgulated.

5) We have found that mutation of some of the putative CDC28 phosphorylation sites is sufficient to alter the cell cycle regulated nuclear localization of a DFHR-SWI5 fusion protein.

Work is currently under way to test whether the amino acid residues found to be differentially phosphorylated in SWI5 prepared from mitotically-arrested cells and from G1-arrested cells are within the consensus phosphorylation sequences of the CDC28 kinase.

Transcription factors involved in the cell cycle regulation of the *HO* gene.

I. Muroff, S. Sockanathan, R. Taba

Our work concerns a cell cycle regulated event which may be triggered by a putative increase in kinase activity which occurs when cells undergo START in the G1 phase of the cell cycle. Here again, the system concerns the regulation of the *HO* endonuclease gene. *HO* transcription takes place very transiently during the G1 phase of the cell cycle as cells undergo START. *HO* activation is dependent on the CDC28 kinase. Though the *HO* promoter is very large and complicated, since the gene is also regulated by the mating type locus and is only expressed in mother cells, it has nevertheless been possible to identify a simple and highly specific regulatory sequence, the CACGA4 repeat, which is responsible for the CDC28 dependence of the promoter during G1. Two genes, called *SWI4* and *SWI6*, are necessary for the biological activity of the CACGA4 repeat.

We have addressed two questions. Do the SWI4 and SWI6 proteins bind in a sequence specific manner to the CACGA4 repeat and, if so, are the complexes so formed dependent on the CDC28 protein kinase? Using a gel retardation assay, we have demonstrated that SWI4 and SWI6 will form specific complexes in vitro on fragments of the *HO* promoter which contain the CACGA4 repeat. Under normal circumstances both SWI4 and SWI6 are needed to form such complexes, but if the concentration of the SWI4 protein is raised by expressing the SWI4 protein from the strong *GAL1-10* promoter, SWI4 can form stable complexes without the participation of SWI6. This effect is not simply an in vitro artefact since the *HO* promoter in vivo also becomes independent of SWI6 under such circumstances. An analysis of the contacts made between SWI4 and DNA suggests that recognition of CACGA4 is made primarily by SWI4. In this case, SWI6 may act by increasing the affinity of SWI4 binding.

The gel retardation assay used to detect the SWI4/6-DNA complexes has also been useful in addressing the question of cell cycle control. Two types of SWI4/6 complex, which differ in their migration rate through an acrylamide gel, can be detected when extracts are made from asynchronous cultures. Both complexes involve binding of proteins to the CACGA4 repeat since their formation can be prevented by the addition of a 100 fold molar excess of synthetic CACGA4 oligonucleotide (i.e. they are compatible). Moreover, both complexes contain the SWI6 protein since their migration through the gel can be further retarded by the addition of anti-SWI6 antibodies. The faster migrating of the two complexes can be detected at all stages of the cell cycle but the slower complex is cell cycle dependent. It is absent in early G1 cells and its formation seems to be dependent on the CDC28 protein kinase. We are at the moment trying to test whether any of the proteins in the complex (presumably SWI4 or SWI6) are themselves substrates for the kinase and whether phosphorylation is responsible for the different electrophoretic migration of the two complexes.

A clue as to whether either SWI4 or SWI6 may be a substrate for the CDC28 protein kinase has come from an analysis of their distribution within the cell at different stages during the cell cycle. Whereas a SWI4 DHFR fusion protein is accumulated in the nucleus throughout the cell cycle, the distribution of the SWI6 protein seems to vary during the cell cycle. SWI6 is confined to the nucleus in G1 cells but increasing amounts of the protein are found in the cytoplasm as cells approach mitosis. When one analyses the distribution of the protein in cells blocked at different stages of the cell cycle due to various cdc mutations, then, just as for the SWI5 protein, SWI6 is found predominantly in the cytoplasm under circumstances where the CDC28 kinase is active but accumulates in the nucleus when it is inactive. This observation suggests that the properties of SWI6 may be regulated by its state of phosphorylation by the CDC28 kinase.

By combining the DNA binding and cytological studies on SWI5 and SWI6, we are now in a position to propose a working hypothesis for how the *HO* gene is only activated as cells undergo START in the G1 phase of the cell cycle. The inactivity of the gene in G2 or mitotic cells may due to the mis-location of both SWI5 and SWI6 in the cytoplasm. As cells enter G1,

both proteins enter the nucleus and bind to the *HO* promoter but the gene is still not activated since the SWI4/6 complex is in an inactive form (because it is unphosphorylated by CDC28?). At START, we propose that the CDC28 kinase is activated and that phosphorylation of one of the components of the SWI4/6 complex causes it to switch conformation to a form which activates *HO*. If we assume that the CDC28 kinase remains active after START, then it may, paradoxically, also be responsible for turning off *HO* by ensuring that neither SWI5 nor SWI6 can accumulate in the nucleus during the S or G2 phases of the cell cycle. An attractive feature of this model is that it explains how a simple change in the acitivity of a protein kinase could give rise to a transient response such as occurs on the *HO* promoter. That is, we may be glimpsing at a mechanism for the transduction of a "step" into a "peak".

It should finally be added that the identification of SWI4 or SWI6 as potential substrates for the CDC28 kinase may represent an important step in our understanding of this kinase. So far, no G1-specific substrates for CDC28 have been identified and without such substrates it will not be possible to address the important question of whether the enzyme which is active in late G1 is functionally different from the enzyme which is active in late G2. In the former case, the enzyme promotes entry into S phase whereas in the latter it drives cells into mitosis.

Publications during 1989

Harashima, S., Miller, A.M., Tanaka, K., Kusomoto, K.I., Tanaka, K.I., Mukai, Y., Nasmyth, K. and Oshima, Y. (1989) Mating type control in Saccharomyces cerevisiae: Isolation and characterization of mutants defective in repression by a1-alpha2. Mol.Cell Biol. **9**, 4523-4530.

Martin NICKLIN — **Group Leader**
Heinz ULLY — **Diploma Student**

AP1 ANTAGONISTS AS TOOLS TO STUDY CELL GROWTH AND DIFFERENTIATION

OVERVIEW

When treated with a particular factor or set of factors, a cell in culture may enter or exit from the cell cycle, change its morphology or behaviour, or begin to differentiate. Such responses reflect the way in which cells *in vivo* adapt appropriately in the long term to new external signals. The transcription of numerous genes becomes activated within ten minutes of the stimulation of cells with external factors. Such genes have been called "immediate early" genes. Some members of this group, in particular those genes of the *fos* and *jun* families, which encode the protein components of the transcription factor AP1, have been proposed to couple short-term cellular stimulation and signal transduction with appropriate long-term responses (Curran,1988). The AP1 proteins are an homologous group of proteins that must dimerize in order to bind to DNA. They have well defined and separable domains for interaction with DNA and for dimerization. In principle, therefore, it is simple to design proteins that lack one or other function of the normal AP1 proteins, and which will therefore interfere with the activity of some or all of the AP1 proteins when expressed *in vivo*.

We are developing proteins that will antagonize the activity of AP1 proteins *in vitro* and *in vivo*, and investigating the refinement of their specificity. By expressing these proteins *in vivo* in a regulatable expression system, we will be able to inactivate AP1 in a controlled manner. We will then investigate whether AP1 activity is required for entry into the cell cycle in fibroblast cell lines or for differentiation in embryonic carcinoma cells.

DESIGN OF PROTEINS THAT INTERFERE WITH AP1 FUNCTION.

Protein antagonists should block the activity of their target proteins either by binding directly to them according to their homologous group, or by preventing access to their

common DNA target-site. We are now investigating both approaches, but only the former is described below.

Proteins Defective in DNA binding.

Mutated Fos Proteins. Fos and Jun proteins have an extended region of homology, with exact conservation of spacing of invariant residues. Within this region lies the DNA-binding domain and the 'leucine zipper', a long alpha-helical segment that permits specific interaction between the Fos and Jun proteins (Turner and Tjian. 1989). We have already shown that simple mutations in the DNA binding domain of Fos can produce defective proteins that interfere *in vitro* with binding of Jun to DNA. These mutated proteins compete stoichiometrically with the unmutated Fos protein in the formation of Fos-Jun complexes, and complexes containing mutated Fos protein do not bind to DNA. It is clear therefore that one good DNA-binding domain per heterodimer is insufficient to permit recognition of the AP1-binding site (ABS). The effect of these mutant proteins is complex *in vivo*. On transient expression in F9 cells, the mutated proteins reduce the transactivation of a 5xABS-dependent reporter gene (5xABS-CAT) by cotransfected wild-type Fos, as we would predict in cells which have very low endogenous AP1 activity. In NIH3T3 cells, with their higher endogenous AP1 levels, the mutant Fos proteins cause a slight increase in the activity of the 5xABS reporter gene, probably by titrating away the endogenous Jun proteins and thus removing repressor from the *c-fos* promoter. Accordingly, we have seen a stronger increase in the activity of a reporter gene linked to the *c-fos* promoter (*fos*-CAT). In a parallel experiment, the unmutated protein was found to repress basal expression from *fos*-CAT as has been previously reported [Sassone-Corsi et al., 1988]. Thus, our interfering Fos proteins, which do not interact with endogenous Fos and thus leave the concentration of active Fos protein uncontrolled, are probably merely perturbing the intricate balance of regulation of Fos and Jun expression *in vivo*.

Grafting Zippers onto Stable Carrier Proteins. Presumably, the mutated Fos or Jun proteins will not be more stable than the wild-type proteins, *in vivo*. To achieve more efficient inactivation of endogenous AP1, and at the same time to remove any other uncontrolled

activities of the parent proteins, we have embarked on an investigation of the effects of expressing the segment of the Jun protein that interacts with Fos (the zipper) as a component of a stabler protein which will accumulate over a longer period within the cell. It has been shown (O'Shea et al., 1989) that isolated zippers are able to interact tightly with one another.

The advantage that the short-lived proteins will have over stable derivatives is that they could be induced and allowed to turn over within a few hours. Stabler proteins will be more likely to achieve the target of complete inactivation of AP1. We will also consider making amino-terminal modifications of the type reported to modulate the half-life of proteins *in vivo* [Bachmair et al., 1986] if necessary.

Manipulation of the Leucine Zipper to Modulate Specificity. This year I began to investigate the detailed interactions that give the leucine zippers of Fos and Jun their specificity. Specificity is supposed to result from the residues that in the helical zipper structure lie adjacent to the central hydrophobic ridge. I was able to predict an interaction which caused the Fos protein to form homodimers and bind at a ABS. I am currently investigating a detailed model of the interaction of two leucine zippers. I would like to use information from this study to design a zipper that would interact strongly and exclusively with wild-type Fos protein. Such a zipper could be incorporated into any of the type of constructions I have mentioned above.

REFERENCES

Bachmair et al. (1986) Science **234**, 179.

Curran (1988) in the Oncogene Handbook (Elsevier), p307

Maekawa et al. (1989) **8**, 2023.

O'Shea et al. (1989) Science **245**, 646.

Sassone-Corsi et al. (1988) Nature **334**, 314.

Turner and Tjian (1989) Science **232**, 1689.

Gotthold Schaffner	**Group leader**
Sissy Ender	**Technician**
Ivan Botto	**Technician**
Robert Kurzbauer	**Technician**

The Service Department offers instant help in many fields to all members of the institute. DNA sequencing and the synthesis and purification of oligo-nucleotides consume the bulk of our time. In addition, the immunology branch of the service department produces, with the help of the animal house staff, rabbit antisera to isolated proteins and various small oligopeptides, synthesized by Ernst Wagner and Christian Plank. The titers of these sera are regularly tested. The Service Department also serves as an in house depository of cloning vectors and primers, as well as providing selected DNA "reagents" (e.g. molecular weight markers) and cloning strategies.

Oligonucleotides

During 1989 we synthesized greater than 800 DNA-oligonucleotides using an APPLIED BIOSYSTEMS "380 B" (a three column synthesizer) and a PHARMACIA "GeneAssembler" using the Phosphoroamidite-technique. A few syntheses with the H-Phosphonate-approach were performed, because its chemistry is more suited for subsequent modification, e.g. Phosphoro-thioate oligonucleotides. Short oligonucleotides <20 nucleotides (e.g. sequencing-primers) were used after ethanol precipitation without any further treatment. The standard technique for the purification of oligonucleotides >20 nucleotides involves the isolation of the oligonucleotide from acrylamide gels.

In late July we began the syntheses of RNA- and 2'-methoxy-RNA- oligonucleotides. Most of the protected monomers were prepared by Dr.Noe's group at the Technical University of Vienna. The deprotection of the synthesized oligonucleotides is sometimes a problem. To shorten the deprotection time, they prepared monomers with phenoxyacetyl- instead of benzoyl- or isobutyryl-residues for base protection. The synthesis of the oligomers themselves required only slight modification of the standard protocol for DNA synthesis : coupling- and oxidation-time were increased and a prolonged detritylation time was used as well, because of the higher stability of the monomethoxytrityl-group.

Sequencing

Most of the oligonucleotides generated by the I.M.P. Service Department are cloned by I.M.P. members and are then sequenced by the Service Department in their plasmid constructs. The DNA prepared from "minipreps" by alkaline lysis or by a "boiling-prep-procedure" is normally of low quality. However, we have optimized the conditions for double-stranded DNA-sequencing from these quick preps. RNase treatment and/or a phenol-extraction is normally not necessary, e.g. with the "boiling-prep-procedure" a precipitation step with alcohol is sufficient for

sequencing. In addition to sequencing cloned oligo constructs, we have also sequenced about 15 kbp of "new" genes and 3 kbp of known genes in which the sequence was found to be incorrectly published. For this class of sequencing, DNA isolated from CsCl-gradients was used. Most of the sequencing was performed with double stranded DNA and primers synthesized for those DNAs ("primer hopping"). About 1500 sequencing reactions were made since January, >95 % with double stranded DNA routinely using the T7-DNA polymerase (PHARMACIA) with the SEQUENASE-protocol (USB) and ^{32}P-dATP.

In 1990 we will most likely use one of the automated sequencers and a robot (Biomek from Beckman) to make sequencing more attractive and more efficient.

Tillman Schuster **Group leader**

Clive Price **Postdoc**

Wilfried Rossoll **Diploma student**

Genetic investigation of the cell cycle of the yeast *Saccharomyces cerevisiae* has revealed many genes which are involved in cell cycle progression and its regulation. Most current knowledge has been gained by the isolation and study of the Cell Division Cycle genes (CDC genes) in the yeasts *S. cerevisiae* and *S. pombe*. This has led to the recognition of two crucial regulatory points in the cell cycle, the so-called START, which is located in late G1, and a point in G2 which is responsible for the transition from the G2 state to mitosis. Involved in the control of these two points are the products of the CDC28 gene of *S. cerevisiae* and its homologue CDC2 in *S. pombe*. However, the biochemical events immediately preceding and following the execution points of these genes remain unknown. Several studies indicate that transcriptional control plays a key role in the cell cycle dependent regulation of genes. Our interest lies in the role differential gene expression plays in the regulation of cell cycle progression. Our working hypothesis is that genes may exist which are expressed only at specific points in the cell cycle, and that their gene products may be involved in the regulation of cell cycle progression. In order to find these genes we decided to isolate all cell cycle regulated genes of *S. cerevisiae* and to test them for their function with respect to the cell cycle. Since we were intending to look for transient expression of transcripts, hybridization techniques seemed to be the appropriate approach. After excluding several practical approaches such as differential hybridization technique or dot blot analysis we chose Northern blot analysis as the appropriate technique. Transcripts are separated on gels according to their sizes. Thus constitutively expressed transcripts may easily be distinguished from cell cycle controlled transcripts, and the sizes of the different RNAs can be determined immediately. We decided to use poly(A+) RNA isolated at different cell cycle stages from both haploid and diploid yeast cultures. In order to screen the entire yeast genome for cell cycle controlled transcripts we use an ordered yeast

genomic library in phage lambda provided by M. Olson (Olson, M.V. et.al., PNAS 83, 7826-7830, 1986). The yeast DNA inserts are ordered according to their HindIII/EcoRI restriction pattern. The whole library encompasses 855 phages with inserts of minimal overlap each containing between six and eleven EcoRI/HindIII fragments. In order not to miss any transcripts each restriction fragment is used separately as a probe.

To date we have been able to screen more than 40% of the library. The analysis of one third of our data has revealed 50 transcripts which show strong cell cycle regulation. Subcloning and sequencing experiments have led to the detection of several genes some of which had been previously characterized. The ribonucleotide reductase gene (RNR1), for example, was identified by searching the available databases for homologies. We found it to be strongly induced in S-phase and it mapped to the fifth chromosome, close to the centromere. We have also identified several genes, for which we have been unable to detect any DNA or peptide homologies. We are currently analysing one gene in our collection, which is strongly induced in anaphase and repressed in other cell cycle stages.

Screening of a contiguous DNA segment with a length of 422 kb has allowed us to make certain general predictions about the transcriptional characteristics of the yeast genome. We believe that the whole genome of a haploid cell, encompassing 14,000 kb, directs the synthesis of 5000 transcripts which are of an average length of 1.8 kb and occur once every 2.8 kb. This suggests that 65% of the yeast genome is transcriptionally active. Further we would expect to identify 550 genes which exhibit some degree of cell cycle regulation, of these perhaps 30% will show a strong cell cycle specific regulation.

Ernst WAGNER	**Junior Scientist**
Berndt OBERHAUSER	**Postdoc**
Christian PLANK	**Diploma Student**
Karl MECHTLER	**Technician**
Helen KIRLAPPOS	**Technician**

Synthesis of transferrin-polycation conjugates for the use as DNA delivery system to living cells

(Ernst Wagner, Matt Cotten, Francoise Laengle-Rouault, Martin Zenke, Hartmut Beug and Max L. Birnstiel)

The inefficient uptake of nucleic acid molecules into cells presents a substantial limitation to the inhibition of genes by antisense oligonucleotides or to the introduction of artifical gene constructs. To overcome this barrier our efforts aim at the adaptation of natural transport systems for delivery of natural or modified oligonucleotides and for high molecular weight DNA. All actively metabolising cells require iron which is supplied as a transferrin - iron complex and taken up into cells by receptor mediated endocytosis. To exploit this efficient mechanism for transport of DNA, conjugates of polycations with chicken or human transferrin were synthesized by chemical ligation via disulfide bonds. Polylysines with different sizes of polymer (about 90, 270 or 450 L-lysine monomers) with or without fluorescent label were used. Furthermore conjugates with protamine, a natural occuring arginine-rich DNA binding protein, were synthesized. All conjugates were purified, characterized and saturated with iron. DNA binding at physiological conditions was confirmed by gel mobility shift assay with Eco RI/Hind III - cut Lambda DNA fragments. The successful uptake into cells , the appearance of fluorescent vesicles and the function of the modified transferrins which can replace normal transferrin in terms of iron import , was shown by Hartmut Beug using avian erythroblast tissue cultures. The new delivery system has been successfully used for the introduction of a large DNA molecule (the pRSVLuc plasmid) into avian as well as human erythroblasts resulting in high level expression of the luciferase gene (Martin Zenke and Matt Cotten); Matt Cotten has also applied this method for the delivery of short modified oligoribonucleotides.

Modified Oligonucleotides

Berndt Oberhauser, Gotthold Schaffner and Ernst Wagner

Small antisense oligonucleotides and ribozymes are important tools for the study of gene regulation and inhibition of genes. Once imported, natural oligonucleotides are subjected to a fast nuclease degradation within a cell. Modified analogues with increased stability, 2'-O - methylated oligoribonucleotides and phosphorothioates, have been synthesized and are investigated. Further modifications at the 3' and/or 5' end of the oligonucleotide chain allow the introduction of fluorescent labels or other signals.

Peptide Synthesis

Christian Plank and Ernst Wagner

Several peptides of biological interest were prepared by solid phase synthesis: signal peptides for the targeting to the nucleus; DNA binding peptides which may lead to improved DNA delivery systems; peptides with a multimeric RNA pol II phosphorylation site as substrates for kinase activity have been synthesized as well as several peptides (and conjugates) for the purpose of generating antibodies. Many of these peptides have been provided with functional groups that enable specific coupling to other compounds. Furthermore the synthesis of partial unnatural structures like branched peptides has been performed.

Nuclear magnetic resonance (NMR) spectroscopy as tool for conformational studies

Berndt Oberhauser, Christian Plank and Ernst Wagner

The spatial structure of deltorphin, a delta-opioid heptapeptide, has been the topic of theoretical molecular mechanic calculations by Helfried Schreiber and Anton Beyer. In order to obtain some data about the real conformation in solution, the peptide was synthesized and its solution in 90% water / 10% deuteriumoxide was investigated by one dimensional (1D) and two

signals) was suppressed by a special presaturation technique. 2D-NMR measurements that detect magnetic transfer from one excited hydrogen to an adjacent one either through the bonds that connect the atoms (coherence effects - COSY, HOHAHA experiments) or through the space (population effects - NOESY, ROESY spectra) enabled the assignment of the individual hydrogens. These experiments together with a series of measurements at different temperatures show evidence for conformational flexibility - as was to be expected with such a small peptide - but also a preference for a characteristic structure (at least in the N-terminal part of the peptide) that fits into the theoretical calculations or may be similar.

Publication during the year.

Wagner, E. , Zenke, M., Cotten, M., Beug, H. and Birnstiel, M.L. (1990) Transferrin-polycation conjugates as carriers for DNA uptake into cells , *Proc. Natl. Acad. Sci. USA* **87** , in press.

Erwin WAGNER	**Senior Scientist**
Gordon KELLER	**Visiting Scientist**
Zhao-Qi WANG	**IMP Postdoc**
Konrad HECKL	**IMP Postdoc**
Adriano AGUZZI	**IMP Postdoc**
Thomas von RÜDEN	**EMBO Postdoc**
Frank HILBERG	**EMBO Postdoc**
Gabor VERES	**DFG Postdoc**
Agi GRIGORIADIS	**MRC Postdoc**
Friedemann KIEFER	**IMP Grad. Student**
Ulrike BURKERT	**IMP Grad. Student**
Uta MÖHLE-STEINLEIN	**IMP Technician**
Marion KENNEDY	**IMP Technician**
Erika SCHELLANDER	**DFG Technician**

Gene function in mammalian development and differentiation

The aims of our research program are to analyze the function and regulation of genes in mammalian stem cell differentiation and development and to study the molecular events leading to tumor formation. For these studies the method of gene transfer is used as a powerful tool in three developmental systems:

I. Transgenic mice are produced by introducing genes into the germ line of mice by microinjection of DNA into fertilized eggs.

II. A complementary overlapping approach uses embryonic stem (ES) cells as target cells for gene transfer to express ectopically foreign genes as well as to inactivate gene function. Genetically altered ES cells are being introduced into mouse embryos in order to analyze the consequences of the altered genotype in chimaeras and their transgenic offspring.

III. To study hematopoiesis we are perturbing normal hematopoietic development through ectopic expression of growth control genes in murine bone marrow cells.

I. GENE EXPRESSION IN TRANSGENIC MICE

c-*fos* transgenic mice as models for analyzing bone differentiation

A. Grigoriadis, K. Heckl, Z.-Q. Wang

We have continued our efforts in collaboration with Karl Schellander (Vet.Univ., Vienna) and Ulrich Rüther (EMBL) to study the function of the proto-oncogene c-*fos in vivo* by analyzing a number of transgenic mice which carry different *fos* fusion genes in their germ line. Our data demonstrate a correlation between the phenotype observed and the type of construct expressed in mice (see publications during 1989). Independent of the promoter and despite high c-*fos* expression in various tissues, mice which express c-*fos* from vectors where the 3´ non-coding part of the gene is replaced by a portion of FBJ MSV including the long terminal repeat (LTR), specifically develop bone lesions which can progress to bone tumors (Fig.1A). In the absence of the 3´ LTR, transgenic mice show either no phenotype (despite high levels of expression) or develop thymic hyperplasias without progression to tumors.

To investigate the pathogenesis of the transgenic mice (MT-c-*fos* LTR) exhibiting altered bone differentiation, we examined by *in situ* hybridization the temporal and spatial expression of c-*fos* during development, in early postnatal life as well as in the bone tumors (Heckl and Wagner, 1989). We found that the onset of the observed phenotype is preceded by exogenous c-*fos* expression and can be attributed to elevated levels of c-*fos* in osteoblasts in the region of perichondrial ossification and to chondroblasts in the epipyhseal cartilage. Within the tumors, only specific areas expressed high levels of c-*fos*. In collaboration with Jörg Schmidt (GSF Munich) we have begun characterizing the c-*fos* induced bone tumors and have established a number of cell lines from them and compared the data to v-*fos* induced osteosarcomas. Preliminary evidence suggests that terminal osteogenic differentiation is blocked within these lines as demonstrated by the lack of osteocalcin expression and by the marked reduction in parathyroid hormone (PTH) responsiveness. All of the lines tested were found to express inducible high levels of the exogenous c-*fos* gene. Interestingly, the endogenous c-*fos* gene is not expressed. Our studies suggest that the c-*fos* gene is a regulator in bone development and that we next have to dissect the cellular and molecular targets of *fos* action.

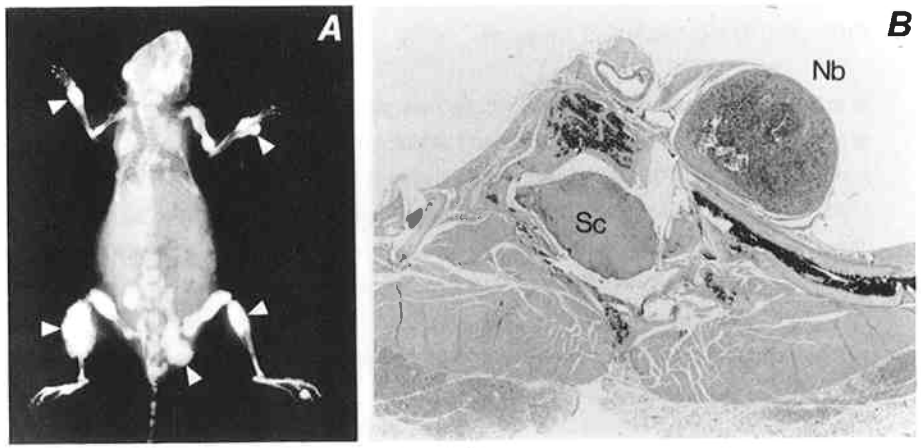


Fig.1 Transgenic mouse models for bone tumors (A) and neuroblastomas (B)

c-*jun* expression in transgenic mice *F. Hilberg*

To investigate the role of the proto-oncogene c-*jun* during mouse development and to study its interaction with c-*fos* we are generating mouse strains overexpressing c-*jun*. We placed the coding region of both the mouse and the human gene under the control of strong enhancer/promoter sequences, such as the human-b-actin promoter, the H2 promoter, the viral RSV and PCMV promoters. The different c-*jun* constructs were introduced into fertilized eggs by DNA-injection and several transgenic lines were obtained. Preliminary RNA analysis from some of the transgenic lines indicates that the transgenes are expressed, however, we have not yet observed a phenotype.

GHF-1 (Pit-1) expression in transgenic mice *G. Veres*

In collaboration with Michael Karin (UCSD), we have generated transgenic mice carrying the gene for the pituitary-specific transcription factor, GHF-1, under the control of different promoter elements. The goal of these experiments is to investigate whether ectopically expressed GHF-1 can activate either endogenous target genes or a co-injected reporter gene, GH-CAT. Currently, we are in the process of analyzing the expression of the transgenes.

A mouse model for human neuroblastoma *A. Aguzzi*

Transgenic mice were generated by microinjection of a construct carrying the Polyoma middle T antigen (mT) under the control of the HSV thymidine kinase (TK) promoter. Two of 4 founder animals developed no phenotype and one died of generalized visceral hemangiomas. The fourth animal, however, allowed the establishment of a transgenic family in which with 100% penetrance all mice suffer from a profound alteration of cell differentiation in the peripheral nervous system (Fig.1B). Four distinct, often synchronously occurring pathologies were identified: 1) Generalized hyperplasia of the ganglia of the sympathetic trunk, 2) paravertebral tumors originating from the sympathetic ganglia, 3) tumors of the

adrenal medulla and 4) in some animals, a focal hyperplasia of the Purkinje cell layer in the cerebellum.

Expression of mT was detected by tyrosine kinase assay (in collaboration with Sara Courtneidge, EMBL) only in the tumors and in the brain. Extensive histological examinations together with neuronal differentiation markers (e.g. synaptophysin and GFAP) allowed us to describe the sequence of events which leads to tumor formation through a progression from hyperplastic and dysplastic lesions. We believe that these animals provide a unique *in vivo* model to study the pathogenesis and cell biology of human peripheral neuroblastoma which is the most common malignant solid tumor in childhood. Although several cell lines are available from human and rodent neuroblastic tumors, no *in vivo* models are available. In order to devise new therapeutic strategies, an animal model in which these tumors would predictably arise is highly desirable.

II. STUDIES USING EMBRYONIC STEM (ES) CELLS

We have continued and initiated several investigations into the use of ES cells as a powerful route for introducing defined genetic information into mice. We are exploiting this system to: 1) study the effect of various growth control genes and oncogenes on stem cell differentiation *in vitro*, 2) analyze dominant effects on growth control in early embryos *in vivo*, and 3) generate mice deficient for specific gene products.

Growth control in ES cells *U. Möhle-Steinlein*

The maintenance of ES cells *in vitro* requires the presence of a soluble factor, myeloid leukaemia inhibitory factor (LIF; Williams et al., 1988 Nature 336, 684-687), which is produced by a variety of feeder cells. In collaboration with Lindsay Williams (WEHI) we were able to generate new ES cell lines by directly explanting blastocysts into LIF-containing medium (MBL-1 to -5), and were able to demonstrate the totipotency of these new cell lines by the

formation of germ-line chimaeras. In the absence of LIF, ES cells differentiate spontaneously in culture. This *in vitro* differentiation seems to be prevented by the expression of various oncogenes, e.g. Polyoma virus middle T antigen (mT), v-*src* as well as by *fos*. The capacity to differentiate is not completely blocked within these ES cells as they can still do so following injection into blastocysts (see below). Studies are currently under way to determine the mechanism by which these oncogenes can influence the growth and regulatory pathways of ES cells *in vitro*.

Dominant mouse mutants produced from mT expressing ES cells

A. Aguzzi, U. Möhle-Steinlein

To study the consequences of modulating tyrosine kinase activity *in vivo*, several ES cell clones expressing mT from retroviral constructs were isolated and used to generate chimaeras. The chimaeras generated with mT-expressing ES cells were found to be arrested in development specifically at midgestation. The abnormal embryos displayed extensive disruption of the blood vessels due to the appearance of multiple cavernous hemangiomas (Williams et al., 1988, Cell 52, 121-131). To investigate the mechanism of hemangioma formation, endothelioma cell lines (End. cells) expressing the mT antigen were derived from these tumors. When these End. cells were injected into mice, chicken and quail embryos they rapidly induced hemangiomas and histological analysis indicated that the majority of the endothelial cells in the hemangiomas were derived from the host tissues (Williams et al., 1989). In collaboration with Werner Risau (MPI, Munich) we could demonstrate that there is no endothelial-specific mitogenic activity secreted by the End. cells and that no induction of host cell proliferation was detectable. We have recently begun to analyze the End. cells for the expression of proteases/protease inhibitors and found a specific induction of u-PA/t-PA activity in some of these cell lines (in collaboration with Roberto Montesano, Geneva). We are currently aiming to provide further support for the causal role of high proteolytic activity in the induction of hemangiomas.

Dominant expression of *fos/jun* genes

U. Möhle-Steinlein, F. Hilberg, Z.-Q. Wang, A. Grigoriadis

To study the function of c-*fos*/c-*jun* in stem cell differentiation and early mouse development, various *fos* and *jun* fusion genes were introduced into different ES cell lines. Several ES cell lines expressing the introduced *fos/jun* genes were isolated and were found to maintain the stem cell morphology when cultured in LIF-containing medium. ES cell lines expressing different amounts of exogenous c-*fos* were used to generate chimaeras following blastocyst injection. A number of chimaeric mice were obtained, some of which developed characteristic chondrosarcomas, and they are presently being analyzed for c-*fos* expression and the molecular and cellular changes caused by the overexpressed oncogene.

Gene inactivation in ES cells by homologous recombination

U. Möhle-Steinlein, F. Hilberg, G. Veres, G. Keller

One of the most powerful tools to study gene function is the specific inactivation of a particular gene. We have generated "inactivation vectors" for c-*fos*, c-*jun* and interleukin-3, all based on the positive/negative selection scheme (Mansour et al. 1988 Nature 336, 348-352 and Fig.2). In addition, in collaboration with Ulrich Rüther (EMBL), we are trying to inactivate c-*fos* by microinjection of targeting constructs without selection. Presently several candidate ES cell clones are being characterized in detail and will be used to generate germ-line chimaeras.

Expression of the human EGF receptor (HER) in EC/ES cells and in transgenic mice *G. Veres*

The EGF-receptor is one of the most extensively studied growth factor receptors to date and a great deal of data are available concerning its structural and functional properties, however, its role in cell proliferation and tumorigenesis remains largely unknown. To address some of these questions we have introduced constructs (some obtained from A. Ullrich, MPI, Munich)

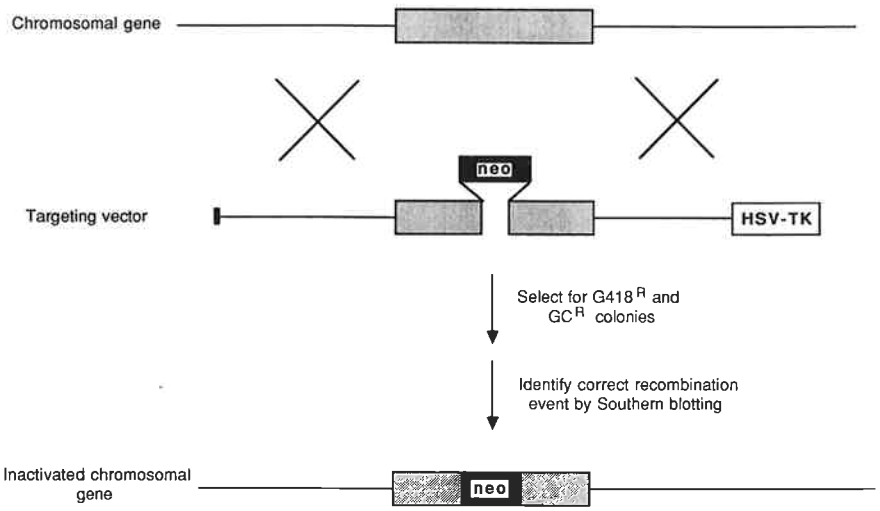


Fig. 2 Scheme for gene inactivation in ES cells by Homologous Recombination

containing the human EGF receptor (HER) into embryonal carcinoma (EC) and embryonic stem (ES) cells as well as into fertilized mouse eggs. F9 EC cells, which express no endogenous EGF-receptors in the undifferentiated state, were transfected with HER constructs. Individual G418- resistant colonies are being analyzed at the RNA level. The expression of functional receptor protein and its effect on the differentiation of F9 EC cells following induction with retinoic acid is also being investigated in the presence and absence of EGF.

Transgenic mouse lines, established by injecting the HER cDNA linked either to the constitutive hydroxy-3-methylglutaryl-coenzyme A reductase (HMG) promoter or the inducible

mouse mammary tumor virus (MMTV) promoter are being analyzed for expression of HER mRNA by Northern blot and RNase protection analysis. In addition, we have recently generated MMTV-HER2 transgenic lines with a mutated form of the HER, HER2, and expression of this transgene is also currently being analyzed.

III. HEMATOPOIETIC STEM CELL DEVELOPMENT

This section describes a preliminary study aimed at purifying hematopoietic stem cells, as well as a series of projects whose goal is to study the effects of expression of oncogenes and growth factor receptor genes on development of the hematopoietic system *in vitro* and *in vivo*.

Approaches towards purification of mouse hematopoietic stem cells

F. Kiefer, G. Keller

The aim of this project is to derive a relatively simple and rapid method for enriching and characterizing stem cells from mouse bone marrow. We have used a competitive repopulation assay (Fig.3) to follow the fate of the stem cells through the various fractionation procedures, as it has distinct advantages over the commonly used radioprotection assay. These include the ability to: 1) distinguish between transient and long term reconstitution, 2) quantitate stem cells following fractionation, and 3) detect relatively low numbers of stem cells.

As a first step we have fractionated mouse bone marrow by percoll-density gradients. Long term repopulating cells were found in a range of densities between 1.073g/cm^3 and 1.081g/cm^3. In addition, bone marrow was separated by adherence to tissue-culture plastic surfaces. Although only 10% of the cells are adherent, these do contain the same number of long term repopulating cells as the non-adherent fraction. The analysis of recipients at different time points following bone marrow reconstitution enables us to distinguish between animals that are transiently reconstituted by intermediate stage precursors, and those which are stably reconstituted by primitive stem cells.

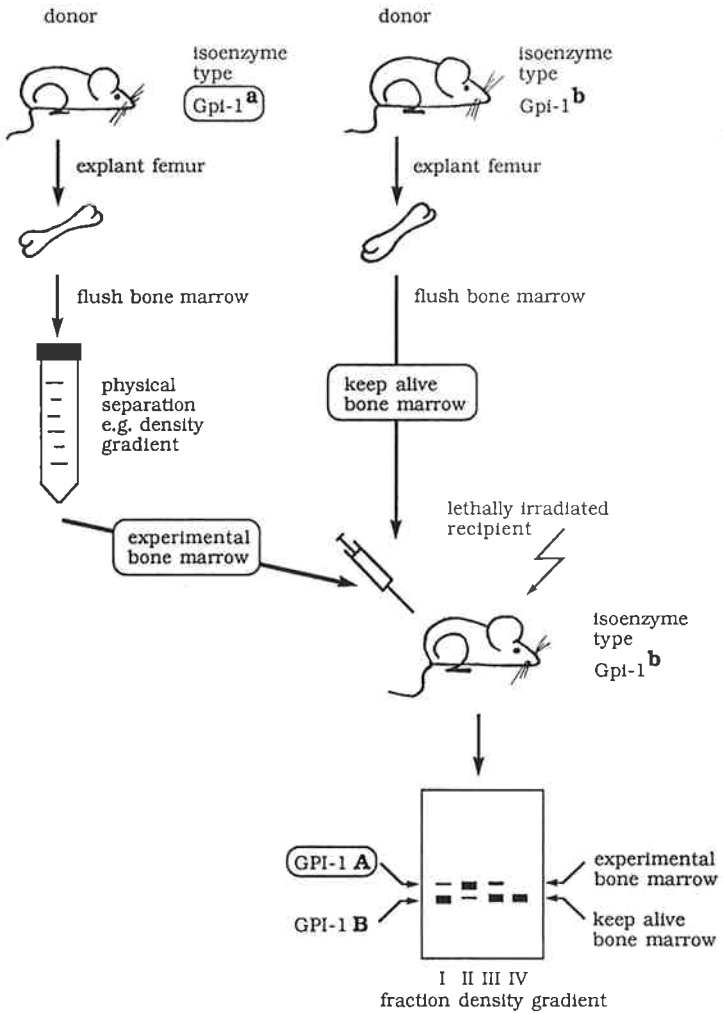


Fig. 3 Schematic diagram to purify and quantitate hematopoietic stem cells

Expression of v-*src* Alters Hematopoietic Development in Bone Marrow Reconstituted Mice *G. Keller, M. Kennedy*

The goal of this project is to study the effects of v-*src* oncogene expression on the development of the hematopoietic system. Bone marrow cells infected with N-TK-*src*, a retrovirus which expresses both v-*src* and the selectable *neo* gene, were used to reconstitute irradiated mice. Expression of the v-*src* oncogene in these animals leads to a

myeloproliferative disorder characterized by splenomegaly, anemia and a shift of hematopoiesis from the bone marrow to the spleen (Keller, G. and Wagner, E.F., 1989). Despite these changes, the precursors derived from these animals appeared normal with respect to their *in vitro* growth requirements and developmental potential.

When spleen cells from these primary recipients were passaged to secondary animals, precursors with an abnormal phenotype could be detected in the spleen and bone marrow. When cultured in methyl cellulose in the presence of interleukin-3 (IL-3) and erythropoietin (Epo) these precursors generated colonies which consisted predominantly of undifferentiated blast cells. A number of these colonies were picked and expanded into continuously growing cell lines in the presence of IL-3 and/or Epo. Analysis of the viral integration sites demonstrated that these cells were derived from clones present in the spleens of both the primary and secondary recipients.

Most of these established lines can be grown in the presence of Epo alone, suggesting that they contain precursors committed to the erythroid lineage. Consistent with this notion is the finding that a proportion of cells (5-30%) in each of the lines tested stained positive for acid benzidine, indicating that they are producing some hemoglobin (in collaboration with Hartmut Beug). In addition, 8 different lines tested stained with an antibody that recognizes most bone marrow cells, including erythroid, but not with antibodies specific for any of the other blood cell lineages. Some of these lines have been adapted to grow in the absence of any added growth factor and under these conditions they produce factors which stimulate the growth of erythroid, neutrophil, macrophage and pluripotential precursors from normal bone marrow.

Infection of mouse bone marrow with a retrovirus expressing Polyoma middle T Antigen (mT)

F. Kiefer, G. Keller

The aim of this study is to determine the effect of expressing mT in normal mouse hematopoietic cells. mT was chosen because it is a strong transforming gene which potentiates the *src*-encoded tyrosine kinase activity and has been shown to have some effects on hematopoietic cells. To achieve efficient transfer of the mT gene we infected bone marrow cells with a retrovirus, pNTK-mT, which expresses both mT and the selectable *neo*^{R} gene. Following infection, approximately 10% of the precursors able to grow in methyl cellulose cultures in the presence of IL-3 were found to be G418 resistant. In addition, cells maintained in liquid culture following infection were shown to express mT and exhibited distinct morphological differences compared to cells infected with the control virus N2. These cells are presently being further characterized and the effect of mT expression is being analyzed in bone marrow reconstituted mice *in vivo*.

Expression of growth factor receptors during hematopoietic development

T. v. Rüden, G.Keller, U. Burkert

The goal of this work is to study the role of growth factor receptors (GF-R) during hematopoietic development by analyzing the biological effects of ectopic GF-R expression *in vitro* and *in vivo*. In particular, we are interested in three questions:
1) Do non-hematopoietic or lineage-restricted GF-Rs function on cells where they are normally not expressed? 2)How does ectopic GF-R expression influence hematopoietic proliferation/differentiation? 3) Is it possible to immortalize early multipotential progenitor cells by supplying additional mitogenic signals via ectopically-expressed GF-Rs?

Recently we reported the retrovirus (NTK-HER) mediated expression of the functional receptor for human epidermal growth factor (HER) on primary murine bone marrow cells (BMC)

and hematopoietic cell lines (von Rüden and Wagner, 1988, EMBO J. **7**, 2749). The HER was chosen because of its structural homology to hematopoietic GF-Rs and the oncogenic capacity of its viral homologue, v-*erb*B, to transform avian erythroblasts. EGF acts synergistically with IL-3 on IL-3-dependent BMC in stimulating DNA synthesis and cell proliferation even under IL-3 saturation conditions. In addition, retroviral vectors expressing various chimaeric HER/v-*erb*B and v-*erb*B wild type genes were constructed. These vectors are characterized by an increasing transforming potential on fibroblasts.
In order to study the biological consequences of ectopic EGF-R expression on hematopoietic development *in vivo*, we have recently reconstituted mice with bone marrow cells infected with NTK-HERc. The capacity of the reconstituting cells to express the EGF-R was confirmed by flow-cytometry. An initial analysis of spleen cells derived from two out of eight short term reconstituted mice indicates that the transplanted mice were indeed reconstituted with donor bone marrow. Expression studies and analysis of long term reconstituted mice are currently in progress.

Publications during 1989

Keller, G. and Wagner, E.F. (1989) Expression of v-*src* induces a myeloproliferative disease in bone marrow reconstituted mice. Genes & Development, **3**, 827-837.

Williams, R.L., Risau, W., Zerwes, H.-G., Drexler, H., Aguzzi, A. and Wagner, E.F. (1989) Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. Cell, **57**, 1053-1063.

Rüther, U., Komitowski, D., Schubert, F.R. and Wagner, E.F. (1989) c-*fos* expression induces bone tumors in transgenic mice. Oncogene, **4**, 861-865.

Heckl, K. and Wagner, E.F. (1989) *In situ* analysis of c-*fos* expression in transgenic mice. In: Current Communications in Molecular Biology: Molecular Genetics of Early Drosophila and Mouse Development. M.R. Capecchi Ed. (1989) pp.117-129 CSH Lab. Press.

Trotter, J., Boulter, C.A., Sontheimer, H., Schachner, M. and Wagner, E.F. (1989) Expression of v-*src* arrests murine glial cell differentiation. Oncogene, **4**, 457-464.

Wagner, E.F., Williams, R.L. and Rüther, U. (1989) c-*fos* and polyoma middle T oncogene expression in transgenic mice and embryonal stem cell chimaeras. In: NATO ASI Series, Vol.H26, Cell to cell signals in mammalian development, ed. S.W. de Laat, Springer-Verlag. pp.301-310.

Rüther, U. and Wagner, E.F. (1989) The specific consequences of c-*fos* expression in transgenic mice. Prog. in NAR and Mol. Biol. **36**, 235-245.

Wagner, E.F. (1989) Oncogenes and transgenic mice. In: Growth factors, differentiation factors and cytokines, ed. A. Habenicht, Springer-Verlag, Heidelberg-New York, pp. 662-683.

Wiestler, O.D., Aguzzi, A., Williams, R.L., Wagner, E.F., Boulter, C.A. and Kleihues, P. (1989) Tumour induction in fetal brain transplants exposed to the viral oncogenes polyoma middle T and v-*src*. In: Perinatal and Multigeneration Carcinogenesis. Ed. N.P. Napalkov et al. IARC, pp.267-274.

von Rüden, T., Keller, G. and Wagner, E.F. (1989) Ectopic expression of growth control genes during mouse hematopoietic development. Leukemia, in press.

Publications submitted

Goralzyk, R., Closs, E.I., Rüther, U., Wagner, E.F., Strauß, P.G., Erfle, V. and Schmidt, J. (1990) Characterization of *fos* induced osteogenic tumors and tumor derived cell lines. Differentiation.

Hengerer, B., Lindholm, D., Heumann, R., Rüther, U., Wagner, E.F. and Thoenen, H. (1990) Lesion-induced increase in nerve growth factor mRNA is mediated by c-*fos*. Proc.Natl.Acad.Sci.USA.

Aguzzi, A., Wagner, E.F., Williams, R.L. and Courtneidge, S.A. (1990) Sympathetic hyperplasia and neuroblastomas in transgenic mice expressing polyoma middle T antigen. Nature.

Boulter, C.A., Aguzzi, A., Williams, R.L., Wagner, E.F., Evans, M.J. and Beddington, R. (1990) Aberrant development and twinning induced by v-*src* in chimaeric mouse embryos. Nature.

IMP SEMINAR LIST 1989

09.01.89 **KLAUS DAMM** Salk Institute, San Diego
"Transactivation by c-*erb*A and v-*erb*A"

13.01.89 **NORMAN ISCOVE** Ontario Cancer Institute
"Hematopoietic Stem Cells"

19.01.89 **ALFRED NORDHEIM** ZMBH, Heidelberg
"The transcriptional control of the human c-*fos* proto-oncogene"

26.01.89 **HENK STUNNENBERG** EMBL, Heidelberg
"Regulation of vaccinia virus transcription and expression of a eukaryotic transcription factor"

02.02.89 **DENIS DUBOULE** EMBL, Heidelberg
"Structural and functional organisation of the murine hox gene network"

09.02.89 **FRANK GROSVELD** NIMR, London
"Regulation of the human β-globin domain"

23.02.89 **JOHN SULSTON** MRC, Cambridge
"The genome map of Caenorhabditis elegans"

28.02.89 **CHRISTIANE NÜSSLEIN-VOLHARD** MPI, Tübingen
"Axis determination in the Drosophila embryo"

03.03.89 **IGOR DAWID** Maryland, U.S.A.
"Molecular analysis of embryonic induction"

13.03.89 **KEITH WILLISON** London
"Molecular analysis of mammalian spermatogenesis with genes from the mouse t-complex"

14.03.89 **JACQUELINE PIERCE** NIH, Bethesda, USA
"Signal transduction through foreign growth factor receptors transfected into IL-3-dependent hematopoietic cells."

15.03.89 **GIULIO DRAETTA** CSH, New York
"The cdc2 protein kinase: Mechanisms of regulation during the eukaryotic cell cycle"

16.03.89 **DIETER GALLWITZ** MPI, Göttingen
"Structural and functional analyses of *ras*-like proteins in yeast and mammals"

23.03.89 **MIKE HALL** Biozentrum, Basel
"Nuclear protein localization in yeast"

30.03.89 **NIC JONES** ICRF, London
"Transcriptional regulation by the adenovirus E1A protein."

31.03.89 **SCOTT EMR** Cal. Tech., Pasadena
"Protein sorting of vacuol proteins in yeast"

06.04.89 **HOWARD RIEZMAN** Biozentrum Basel
"Receptor-Mediated Internalization of the Yeast Pherome α-Factor".

13.04.89 **MOSHE YANIV** Institut Pasteur, Paris
"The mouse *jun* genes family"

11.05.89 **PETER GRUSS** MPI, Göttingen
"Murine developmental control genes"

29.06.89 **RON LASKEY** Cambridge University
"Rebuilding the cell nucleus: DNA replication *in vitro*"

05.07.89 **KAROLY NIKOLICS** Genentech
"The luteinizing hormone receptor: An unusual member of G-protein coupled receptors"

06.07.89 **SUSAN GASSER** Lausanne
"Reconstitution of DNA Loops at Yeast Mating Type Loci"

13.07.89 **BERNARD FORGET** Yale University
"Expression of normal and mutant human globin genes in transgenic mice"

14.07.89 **RIK DERYNCK** Genentech, Inc.
"The TGF-beta family of growth and differentiation factors"

03.08.89 **SARA COURTNEIDEGE** EMBL
src family tyrosine kinases in polymoma virus transformation and normal growth control.

07.09.89 **ALLAN BALMAIN** Beatson Inst., Glasgow
"How do chemicals make genes make tumours?"

11.09.89 **RON REEDER** Fred Hutchinson, Seattle
"Mechanisms of initiation and termination for RNA polymerase I"

12.09.89 **BOB PERRY** Fox Chase Cancer Center
"The novel architecture of mouse ribosomal protein promoters"

18.09.89 **MIKE SHEPARD** Genentech
"The role of receptor tyrosine kinases in tumor progression"

18.09.89 **JOSEPH NEVINS** HHMI, North Carolina
"Mechanisms of Trans-Activation of Transcription"

21.09.89	**FRANCO TATO**	Rome
	"Major mechanisms involved in the block of differentiation of myoblasts transformed by viral oncogenes"	
28.09.89	**MARTIN HOOPER**	Edinburgh
	"Embryonal stem cells and designer mice"	
02.10.89	**MARTIN RAFF**	University College, London
	"Cell diversification and differentiation in the mammalian CHS"	
12.10.89	**J. HOEIJMAKERS**	Rotterdam
	"Genetic dissection of mammalian DNA repair: from yeast to xeroderma pigmentosum and reverse"	
19.10.89	**MANFRED SCHWAB**	DKFZ, Heidelberg
	"Molecular Pathology of Human Neuroblastoma"	
23.10.89	**WILLIAM BROWN**	Oxford University
	"Human chromosomes and their telomeres"	
02.11.89	**MEL GREAVES**	Chester Beatty, London
	"Cell interactions and gene expression in haemopoiesis"	
09.11.89	**PETER HERRLICH**	Karlsruhe
	"Mechanisms in tumor promotion and progression"	
16.11.89	**FRITZ THOMA**	Zurich
	"Formation of chromatin structures in yeast"	
23.11.89	**LEE SILVER**	Inst. Pasteur, Paris
	"Mouse t haplotypes and sperm differentiation - molecular and genetic approaches"	
07.12.89	**MIGUEL BEATO**	Marburg
	"Regulation of MMTV transcription by steroid hormones"	

RESEARCH INSTITUTE OF MOLECULAR PATHOLOGY (I.M.P.)
2nd INTERNATIONAL CONFERENCE ON

"GENE CASCADES AND SECOND MESSENGERS IN CANCER"

organised by: Hartmut Beug, Max L. Birnstiel, Meinrad Busslinger, Kim Nasmyth (Vienna) and Mike Waterfield (London).
Conference Manager: Judith Nicholls.

The Conference is to review the chain of molecular events which lead to reprogramming of the eukaryotic cell in normal development and in malignancy. The invited speakers include:

P. Baeuerle (Cambridge,USA)
R. Bravo (Heidelberg)
P. Chambon (Strasbourg)
T. Curran (Nutley, N.J.)
R. Eisenman (Seattle)
E. Gateff (Mainz)
T. Graf (Heidelberg)
E. Harlow (New York)
D. Houseman (Cambridge,USA)
E. Kandel (New York)
H. Land (London)
J. Maller (Denver)
J. Massague (Worcester, MA)
K. Matsumoto (Palo Alto)
F. McCormick (Emeryville)
S. McKnight (Seattle)
S. McKnight (Baltimore)
W. Moolenaar (Amsterdam)
E. Nigg (Lausanne)
P. Nurse (Oxford)
R. Nusse (Amsterdam)
P. Parker (London)
L. Philipson (Heidelberg)
G. Sprague (Eugene)
K. Tatchell (Raleigh)
J. Thorner (Berkeley)
W. Timberlake (Athens,USA)
R. Tjian (Berkeley)
N. Tonks (Seattle)
R. Treisman (London)
B. Vennström (Stockholm)
R. Weinberg (Cambridge,USA)
K. Yamamoto (San Francisco)

The Conference will be held in the Vienna International Center and will start on the afternoon of May 20th and finish the evening of May 22nd.

A Registration Fee of $75 will be charged, but if need be will be waived for PhD students if their application is accompanied by a letter from their supervisor.
A number of stipends for subsistence or travel of (maximally) öS 3.000,- will be available on application, with first preference given to PhD students.
Information regarding hotels will be sent when you have registered.
Registration Fee includes Lunches, Coffees and Farewell Party.

Registration closes March 15th 1989

There is no application form. Just write and send Registration Fee to:
Prof. Max L. Birnstiel, I.M.P.,
Dr. Bohr-Gasse 7,
1030 Vienna, Austria.