Scientific Report 1990

I.M.P.

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Impressum

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Preface

When I took on the job of organizing the I.M.P. in 1986 my brief was "to establish and maintain a center of scientific research excellence in the biological sciences, especially for the investigation of the molecular mechanisms of oncogenesis". By the end of 1990, 2 3/4 years after the Grand Opening of the I.M.P., the I.M.P. has reached its final size and, I dare say, the goal set out by the sponsors has been reached. The I.M.P. has now 122 employees, amongst them 90 faculty, postdocs, PhD-students, diploma students and technicians, 19 employees in support of science, such as the graphics department, the library, the workshop, the media and wash kitchen, and finally there are 13 employees in the administration, bookkeeping, purchasing department, etc.

It has always been our intention to establish close ties with the University and this will be made easy in the future since the University of Vienna is in the process of building a very large complex adjacent to the I.M.P. which will house the Institutes of General Biochemistry, Biochemistry, Molecular Biology, Microbiology and Genetics, and Molecular Genetics. Our university colleagues hope to move into the new housing by Spring 1992. But even without this physical closeness, the I.M.P. has already many PhD-students and diploma students and in this way gives Austrian undergraduates and graduates an opportunity, by working towards their theses, to learn to use the modern tools and procedures of molecular genetics. Indeed, we come to know many of the undergraduates already at a very early stage, during their 4 week practicals (Wahlbeispiele). In the winter term 90/91 the I.M.P. provided for the first time a well attended lecture course where the wider issues of the experimental work carried out at the I.M.P. were addressed.

Another activity in which the presence of the I.M.P. here in Vienna makes itself felt is the yearly conference which in 1990 was entitled "Genetic Transfer and Alteration in Biology and Medicine" (for list of speakers see page 63 of this booklet). These conferences seem to be very popular and attracted again of the order of 700 participants, a fair number of them from the neighbouring East European countries. Using the advanced techniques of genetical engineering and animal model systems, the I.M.P. was not only of interest to our colleagues, schools and high school teachers, it was also a focal point for the "greens". This necessitated the hiring of a scientist whose sole role is to communicate with the outside world on these issues (see report page 55). We approached our critics in an open way and supplied all information freely to anyone interested which led to more fruitful discussions. And, after much work in the public sector, we should now, touch wood, be able to concentrate on scientific research without further distraction.

The present booklet has been written not only to inform the educated general public but also our colleagues in Austria and abroad. When studying the document the reader will soon determine, that much progress has been made on a whole range of issues. The contributions of Adrian Bird's will be the last to appear in this booklet since Adrian has decided to accept the prestiguous Buchanan Chair at the University of Edinburgh as of Fall 1990. His group has been replaced by two Junior groups headed by Lisa Ballou coming from the Miescher Institute at Basel and Andreas Weith coming from the German Cancer Research Center (DKFZ). Ballou will continue her studies on protein kinase cascades while Weith will concentrate on the characterization and isolation of anti- or tumor suppressor genes in Man. At the end of 1990 the I.M.P. also saw the first wave of students and postdocs leaving the I.M.P. after three years of employment. We wish them all much success and contentment in their activities at their new working places.

Max L. Birnstiel

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I. Receptor-mediated Endocytosis of DNA into Cells

Transferrin-polycation mediated introduction of DNA into human leukemic cells: stimulation by agents which affect the survival of transfected DNA or modulate transferrin receptor levels.

Matt Cotten, Ernst Wagner, Françoise Laengle-Rouault and Max L. Birnstiel

We have pirated natural receptor-mediated endocytosis events to deliver DNA to cells. Eukaryotic cells obtain iron complexed with the carrier protein transferrin which is internalized after binding to a specific receptor (reviewed by Huebers and Finch, 1987). To take advantage of this rapid and widespread internalization event, we have created human transferrin molecules which retain their ability to bind the transferrin receptor and are modified by the addition of a nucleic acid binding domain (either protamine, synthetic protamine, polylysine, polyarginine, or histone H4). Complexes of polycation-transferrin and DNA, when supplied to cells displaying the transferrin receptor, are internalized, resulting in the expression of genes contained on the DNA (Wagner et al., 1990; Zenke et al., 1990, Cotten et al., 1990). We have termed this DNA transfer method *transferrin*fection (Wagner et al., 1990).

Initial studies demonstrated the utility of *transferrin*fection with chicken erythroid cell lines and primary hematopoietic cells (Zenke et al., 1990). To adapt the procedure for human cell lines, we sought to enhance the level of *transferrin*-fected DNA in several different ways. **First**, we included chloroquine during *transferrin*fection, a com-pound known to raise lysosomal pH and inhibit lysosomal hydrolytic enzymes (Luthman and Magnusson, 1983). Using this drug was an attempt to increase the proportion of DNA reaching the nucleus unscathed and available for transcription. We find that choroquine is an absolute requirement for human cell *transferrin*fection, while chicken cell *transferrin*fection is enhanced by chloroquine but does not require the agent. The function of chloroquine in human *transferrin*fection remains a minor puzzle. We have tested a variety of other lysosomotropic agent (monensin, ammonium chloride, methylamine, methionine methyl ester) without finding a functional substitute for chloroquine. **Second**, we subjected the cells to conditions which would be expected to upregulate transferrin receptor levels and to enhance *transferrin*fection of nucleic acids. We find that pretreatment of K562 cells with desferrioxamine (an intracellular iron chelator) or cobalt chloride (a promoter of heme degradation) produces 20-fold enhancements of *transferrin*-fected gene activity (Cotten et al., 1990).

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Antibody-polylysine and rgp120-polylysine conjugates that are useful for the transfection of DNA into CD4+ cells.

Matt Cotten, Ernst Wagner and Max L. Birnstiel

We would like to expand our repertoire of ligand-receptor pairs which we might use for targeted delivery of nucleic acids. Receptor specific antibodies are widely available, therefore, we are testing the applicability of our nucleic acid delivery scheme to antibody-polylysine conjugates. A related approach involves derivatizing viral proteins that are known to be involved in the viral entry. Certainly viruses have refined strategies for delivering nucleic acids to mammalian cells. It is possible that the receptor-ligand interactions used by a virus to gain cellular entry contain additional properties, such as membrane disruption, or suitable intracellular routing that might result in improved uptake of associated nucleic acids.

The surface protein CD4 is involved in the interaction of T-helper lymphocytes with MHC class II molecules on antigen-presenting cells. HIV 1, considered to be the primary causative viral agent of AIDS, gains entry into T cells after an initial interaction of its surface glycoprotein gp120 with the CD4 molecule (reviewed in Robey and Axel, 1990). For certain applications, it might be useful to deliver antiviral nucleic acids to the same cells susceptible to viral infection. Furthermore, the CD4 molecule has been studied in detail; antibodies which recognize the molecule are available as well as a recombinant form of the gp120 (Lasky et al., 1987a, 1987b).

We have prepared polylysine derivatives of both an antibody which recognizes the CD4 molecule (OKT4A) and of rgp120 (supplied by Timothy Gregory, Genentech). Both of these conjugates function to deliver a test gene (*P. pyralis* luciferase) to CD4+ cells. The antibody-polylysine conjugate (like human transferrin-polycation conjugates) requires the presence of chloroquine during transfection for subsequent DNA expression. However, the rgp120-polylysine functions to deliver active DNA without chloroquine, suggesting an alternate intracellular routing of the polycation/DNA complex.

We discovered a potentially useful aspect of polycation/ligand transfection during the course of our gp120 studies. Certain preparations of the polylysine-gp120 conjugates that had low polylysine content were inactive for DNA delivery. We surmised that the fractions contained sufficent gp120 for binding to the cell but insufficient polylysine for ade-quate DNA condensation. Addition of free polylysine to inactive gp120-poly- lysine fractions produced material which was now active for DNA delivery. This indicates that the polycation serves two functions: 1. To attach the ligand to the DNA molecule. 2. To condense the DNA into a form amenable to endocytosis. See Ernst Wagner's report for additional details.

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II. Mechanisms of Posttranscriptional Control

Nucleo + cytoplasmic transport of histone mRNA

Richard Eckner and Wilfried Ellmeier

In mammalian cells mature mRNA is predominantly found in the cytoplasmic compartment, whereas pre-mRNA is confined to the nuclear compartment (1). Two different models can be envisaged to explain this selective export of mature transcripts. In the first model, mRNA processing (comprising splicing and 3' end formation) and transport are uncoupled from each other and are independent but sequential events. Certain features, specific to processed mRNA, are recognized by the cellular RNA export machinery and stimulate mRNA transport to the cytoplasm. In the second model, mRNA processing and transport are tightly linked with each other, in its extreme, the processing apparatus (e. g. snRNPs) and the transport apparatus are identical. If the second model were correct, one would expect to see a direct correlation between the efficiency of processing and transport. The basis for the selective retention of pre-mRNA in the nucleus is not fully understood. In the case of intron containing transcripts, it has been shown that splice site recognition by snRNPs is an important factor for nuclear retention (2,3).

We were interested in testing the above two models of mRNA export and in defining sequences which promote or inhibit mRNA transport. In order to keep the system simple, we mainly concentrated on histone transcripts which only undergo 3' end processing but no splicing. As experimental system we employed HeLa and Cos1 cells in which we introduced via transient transfection assays various recombinant constructs. Cells were fractionated into cytoplasmic and nuclear RNA fractions and the RNA was quantitated by S1 mapping. By comparing signal intensities of nuclear and cytoplasmic RNAs we jugded about the efficiency of RNA export. Typically, for our globin reference mRNA less than 10% of the signal was nuclear.

In a first series of experiments, we made use of the bacterial neomycin resistance gene. Since this gene is of bacterial origin it should not contain sequences in-

fluencing mRNA export in mammalian cells. When inserted into an expression vector lacking defined processing signals, about 50% of the neo mRNA signal is nuclear. Therefore, this transcript is to a considerable extent but not completely transport deficient. Some splicing and polyadenylation occurs in this transcript and is directed by cryptic signals present on plasmid sequences downstream of the neo gene. Fusion of either a globin polyadenylation signal or a histone 3' end formation signal to the neo gene strongly enhances neo mRNA transport. In both cases more than 90% of the signal is cytoplasmic and less than 10% is nuclear. This result demonstrates that 3' processing signals can act as sequences stimulating mRNA export. This notion could also explain the transport phenotype of the parental neo construct where the cryptic processing signals might be responsible for its residual transport capacity.

The above results, however, do not allow to distinguish between the two models of RNA transport mentioned in the introduction. We therefore constructed a set of histone genes each of it having a cis – acting, selfcleaving ribozyme fused to its 3' end. The various constructs differed in their terminal palindrome structures which become located at the 3' end of the transcripts after "ribozyme – processing". 3' end formation of these mRNAs occurs independently of U7 snRNP action and hence is uncoupled from the cellular processing machinery. If the first model for RNA export is correct, the ribozyme cleaved RNA with a wild type palindrome at the 3' end should be exported as efficiently as an RNA processed by U7 snRNP. However, transport of this mRNA is clearly inhibited as only about 60% of it reaches the cytoplasm, 40% remains in the nucleus. In addition, closer examination of the sequence requirements for this transport phenotype revealed that the wildtype palindrome had to cooperate with a region within the histone coding body in order to exhibit some transport. This extended sequence requirement stands in marked contrast to the U7 snRNP mediated processing and transport reaction which only depends on sequences at the extreme 3' end. We therefore concluded that the second model is correct and that processing and transport are tightly coupled events. (The semi-permissive transport phenotype of the ribozyme cleaved mRNA has most probably something to do with the binding of a ribosome associated factor to the 3' terminal histone palindrome; our unpublished results). Even more drastic transport mutations are represented by the ribozyme processed histone mRNAs exposing mutated palindromic structures at their 3' ends. In these cases, more than 90% of the signal is nuclear. Interetingly, these transcripts are all metabolically quite stable. The mRNAs with altered 3' ends represent a new class of transcripts accumulating in the nucleus: in contrast to actively retained pre-mRNAs containing introns, ribozyme processed RNAs remain passively trapped in the nucleus due to their complete lack of export governing sequences. Thus, active and passive principles can contribute to nuclear accumulation of RNAs.

In summary, our analysis demonstrates that 3' end formation signals strongly stimulate mRNA export and that, at least for histone mRNAs, a tight coupling between processing and transport exists. This work therefore suggests that snRNPs may not only be involved in processing of RNAs but also in their export to the cytoplasm. Our results also provide an explanation for the splicing independent transport observed by Legrain and Rosbash (2) and Chang and Sharp (3). The strategy of using ribozymes for the analysis of RNA transport has a considerable potential. It could also be applied to study the transport of polyadenylated or small nuclear RNA or even of pol I and pol III transcripts.

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Regulation of Histone pre mRNA 3' Processing

Ingrid Hoffmann

Histone gene expression is regulated both during and after transcription (1), with the turnover of histone messenger RNA and the regulation of its 3' processing being the principal factors that determine the size of the histone mRNA pool during the cell cycle. The mature ends of the replication-dependent histone mRNAs are generated during 3' processing by endonucleolytic cleavage of a large primary transcript (2). This reaction depends on a highly conserved stem-loop structure also included in the mature RNA, and a purine-rich sequence lying downstream within the spacer transcript (3). Three factors involved in this reaction which act in trans have been identified: the U7 snRNP (4), the heat-labile factor (5), and the hairpin-binding factor (6).

We were able to show how the U7 snRNP participates in the regulation of histone pre mRNA 3' processing during the cell cycle of mouse C3H10T1/2. The 5' sequences of the U7 snRNA that hybridize with the downstream spacer motif during 3' processing are occluded in the G0 stage of the cell cycle and thus not accessible to micrococcal nuclease treatment. During S phase, however, when histone mRNA synthesis is at its peak, the 5' end is exposed and free to interact with histone pre-mRNA. Serum stimulation experiments of G0 arrested cells show that the appearance of the heat-labile factor and the free 5' end of U7 snRNA occur simultaneously. Both seem to be late events in the signal cascade that drives cells from G0 into the cell cycle and proliferation after stimulation by serum. Preliminary data show that the heat-labile factor which itself is active in S-phase but down-regulated in G0 modulates the accessibility of the U7 snRNA 5' end (7).

The capacity for 3' processing of histone H4 pre-mRNA is lost following the differentiation of rat L6 myoblasts to myotubes. In collaboration with D. Larson, P. Zahradka and B. Sells (University of Guelph) we could demonstrate that the activity of the heat-labile factor disappears during myotube differentiation and that the 5' terminal sequences of the U7 snRNA become occluded (8). Thus, differential myotubes exhibit characteristics similar to the 3' processing of histone H4 mRNA in G0 cells, suggesting that a common regulatory mechanism may be involved in reducing the cellular pool of histone mRNA during the cell cycle and following terminal differentiation.

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Biochemistry of Histone RNA 3' End Formation

Alain Vasserot and Karim Tabiti

Among post-transcriptional events, which primarily account for the global regulation of histone gene expression, 3' end formation and mRNA degradation appear to be the major events determining the cytoplasmic abundance of the histone message. 3' end formation involves an endonucleolytic processing of a large primary transcript and requires two sequence motifs on the precursor which are a hallmark of the replication-dependent histone pre-mRNAs: a highly conserved stem-loop structure located immediately upstream of the cleavage site and a downstream, purine-rich, spacer element (reviewed by Birnstiel and Schaufele, 1988).

Like many other RNA maturation events, histone RNA 3' end formation necessitates a snRNP as principal trans-acting factor. Sequencing the RNA moiety of the murine U7 snRNP (Cotten et al., 1988) has reinforced previous evidence that the U7 snRNA terminal sequences are engaged in base-pairing with the downstream spacer element of the histone precursor during the processing reaction (Schaufele et al., 1986). It has been recently shown that the accessibility of the terminal sequences of the U7 snRNA changes during the G0 to S-phase transition, suggesting tht duplex formation with the histone precursor is a major regulatory event (Hoffmann and Birnstiel, 1990).

Histone 3' processing also requires a factor which is completely inactivated at moderate temperature (Gick et al., 1987). The heat-labile factor is cell cycle regulated (Lüscher and Schümperli, 1987) and appears to act directly on the U7 snRNP to activate it by rendering the terminal sequences of the U7 snRNA accessible to base-pairing with the his-tone precursor (Hoffmann and Birnstiel, 1990).

The third protagonist characterized so far is a hairpin binding factor [HBF] (Vasserot et al., 1989) which specifically recognizes the histone stem-loop structure and, thereby, provides another anchor point between the pre-mRNA and the processing machinery. Prevention of this interaction does not completely abolish processing, yet results in a 80-90% decrease in the efficiency of the reaction. Thus, a basal level of processing seems to be ensured by the sole base-pairing interaction between the precursor and the U7 snRNA whereas the interaction between the HBF and its histone target site enhances the reaction by as much as an order of magnitude.

We have undertaken the purification of the processing apparatus with the aim of providing purified components which could be used in reconstitution experiments in order to understand at a molecular level how the cleavage of the precursor occurs. To date, the U7 snRNP has been highly purified by eight different fractionation procedures. Strikingly, these purified particles are still able to support full efficiency of processing, suggesting that both the heat-labile factor and HBF are part of a large entity (> 300 kDa) scaffolded around the U7 snRNA. Nevertheless, on Mono Q column, both the heat-labile factor and the HBF partition into a (major) U7 snRNP-associated pool and into (minor) pools which are free of any detectable U7 snRNA. By this criterion, the heat-labile factor and the HBF behave like several snRNP-specific proteins which are found both in mature snRNP particles and in unassembled nuclear pools (Feeney and Zieve, 1990). Fractions originating from the HBF pools were used in North western analysis and revealed the presence of a 42 kDa protein which specifically reacts with a short labelled RNA encompassing the HBF target site. The heat-labile factor is currently being purified.

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Peptide fragments of histone H1 variants

Harald Kandolf, Christian Plank and Rosamund Smith

In addition to sealing the nucleosomal core, histone H1 is critical for the higher order formation of chromatin. The widely observed variations in chromatin condensation may be due to regional differences in the type and amount of histone H1. Thus, histone H1 might define the transcriptional potential of a cell by stabilizing particular patterns of gene differentiation. Moreover, it is still an open question whether the polymorphism among the H1 histones is functionally meaningful or merely incidental.

We are interested in investigating the major histone H1 variants of Xenopus laevis (H1A, H1B , H1C) and the extreme variant B4, which are present in a tissue specific pattern. In our approach we plan to inhibit translation of the histone H1 message of the single subtypes by injecting antisense oligoribonucleotides and ribozymes into fertilized eggs. The effect on the expression of oocyte type genes will be studied.

We decided to generate antibodies as a basic tool for analysis. Due to the high degree of homology it did not appear promising to use whole proteins for immunization. Instead we chose to use short peptides as antigens. We selected the N-terminal 12 peptide motifs of each of the variants and synthesized these sequences by solid phase synthesis in colaboration with E.Wagner and C.Plank. As part of our collaboration with Eva and Mark Dworkin (Ernst-Boehringer-Institut Vienna) we synthesized the C-terminal peptide (12mer) of the protein B4 from Xenopus laevis, which has histone H1 like features and is restricted to early developmental stages (1). Soybean trypsin inhibitor (STI) was chosen as carrier protein. The STI-peptide conjugates were taken to immunize rabbits. ELISAs (performed by I.Botto) demonstrated the B4 sequence to be most immunogenic, eliciting a high antibody titer very quickly. Several boosts with pure peptide raised the antibody titer of the H1 sera to a high level as well. In initial tests using western blots and in situ immunofluorescence the resulting sera detected the B4 and H1A protein specifically and properly without any purification steps. The sera against the minor variants H1B and H1C are currently being tested.

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The Senior Scientists

Oncogene function and cooperation in chicken and mouse neoplasms

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Introduction

Our group has an ongoing interest in studying mechanisms of oncogene function and oncogene cooperation in the generation of avian leukemia. Together with the group of Martin Zenke (who has been promoted to a Group leader position in 1990) we focus 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. We are currently focusing on the function of the v-*erb* A oncogene in erythroid differentiation. For this ,we use retrovirus vectors that express v-*erb* A in combination with a conditional transforming oncogene that can be switched on or off at will (by temperature; (ts *sea*) or by ligand (c-*erb* B/EGF-receptor).

Apart from our continuous interest in this particular system, 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 (see report of M. Zenke), we have initiated work in several related areas. Firstly, we are trying to determine if and to what extent repression of erythrocyte-specific genes by v-*erb* A (see report 1989 and M. Zenke 1/2s report) is relevant to the leukemic phenotype. For this, these genes are being introduced into suitable retrovirus vectors and then re-expressed in v-*erb* A containing leukemic erythroblasts (H. Beug and I. Leitner in collaboration with S. Fuerstenberg and B. Vennstroem, Stockholm). Secondly we have continued our efforts to elucidate the role of v- and c-*erb* A – phosphorylation for their function in erythroid differentiation, again using the conditional oncogene approach (with C. Glineur and J. Ghysdael, Lille). Thirdly, we are trying to resolve the potential role of endogeneous nuclear hormone receptors in erythroblast differentiation (c- erbA; retinoic acid receptor, estrogen receptor) and to compare these effects to those observed when the respective receptors are overexpressed like v-*erb* A (Ch. Schroeder).

In an attempt to use conditional oncogenes to study transformation of epithelial cells (carcinogenesis) we have employed a mammary epithelial cell system which retains many features of primary mammary gland cells (Reichmann et al, 1989). Ligand-dependent oncogenes; that is, c-*erb* B/EGFR and v/c-*erb* B chimeras (in collaboration with T. v. Rüden, IMP and Axel Ullrich, Munich, FRG) and chimaeric estrogen receptor-*fos* and estrogen receptor-*myc* proteins (in collaboraton with M. Busslinger's group at the IMP and M. Eilers, Heidelberg) are introduced into these epithelial cells to study the potential effects of such 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).

I. c- and v-*erb* A

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

Christian Schroeder, Ulrike Fuhrmann and Hartmut Beug

Last year, we reported that v-*erb* A caused an outgrowth of immature, erythroblast-like cells from infected bone marrow. Individual clones of these v-*erb* A infected erythroblasts ceased to proliferate and disintegrated after 9 to 18 divisions while erythroblast clones transformed by the tyrosine kinase oncogene v-*sea* grew for 25 to 40 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 *sur*vival. 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. The conclusion that v-*erb* A induces outgrowth of erythroid progenitors due to a different mechanism than kinase oncogenes was confirmed by a detailed analysis of several v-*erb* A/ts v-*sea* transformed erythroblast clones which exhibited a lifespan and differentiation phenotype very similar to v-*erb* A-erythroblasts, when their ts v-*sea* oncogene was switched off by cultivation at the non-permissive temperature, but exhibited a prolonged lifespan and differentiation phenotype typical for ts v-*sea*-erythroblasts at the permissive temperature (Schroeder et al, 1990).

Functional significance of v-erb A-phosphorylation

Hartmut Beug, in collaboration with Corinne Glineur and Jacques Ghysdael, Lille, France.

Previous work by J. Ghysdael's group had established that both v-*erb* A and c-*erb* A proteins exhibit phosphorylation on serine (Ser 28, Ser 29) which is enhanced by either cAMP dependent kinases or protein kinase C, both *in vitro* and *in vivo* (Goldberg et al, 1988). In 1989, we reported that mutation of these sites to alanines (AA-v-*erb* A) yielded a protein that was not phosphorylated at these sites and essentially devoid of biological activity. We have now shown that AA-v-*erb* A causes neither an arrest of erythroid differentiation and change of *in vitro* growth properties of the cells nor a suppression of erythrocyte-specific gene transcription. In contrast, a v-*erb* A protein bearing threonine residues at positions 28 and 29 (TT) exhibited basal, but not drug-enhanced phosphorylation in cells and exhibited partial biological activity being able to partially arrest differentiation, but not to alter *in vitro* medium requirements. A construct in which the alanines of AA-v-*erb*A were converted back into serines (SS* *erb* A) behaved like a wild type v-*erb* A protein in every respect, showing that the AA mutation was indeed responsible for the mutant phenotype seen (Glineur et al,1990).

We also showed in 1989 that the protein kinase C inhibitor H7, when used at concentrations sufficient to completely prevent *erb* A phosphorylation in erythroblasts, was able to strongly reduce the biological activity of v-*erb* A and TT-*erb* A in erythroblasts, inducing both differentiation and requirement for complex growth media. In contrast, H7 did not affect proliferation or differentiation of erythroblasts containing either no v-*erb* A or the nonphosphorylated AA-v-*erb* A protein. These studies have now been completed, showing that H7 also relieves the repression of erythroid-specific genes (CA II, Band 3) by v-*erb* A while not affecting expression of these genes in erythroblasts containing no or AA-v-*erb* A. Our results thus indicate that phosphorylation of v-*erb* A does indeed modulate its function as a transcriptional repressor (Glineur et al, 1990). We are currently analysing the significance of c-*erb* A-phosphorylation for its function in erythroblasts, concentrating on both the Ser 28/29 sites also present in v-*erb* A and on a highly conserved casein kinase II site at Ser 12, using appropriate mutants.

Modulation of erythroid differentiation by ligand-dependent transcription factors

Christian Schroeder, Martin Zenke and Hartmut Beug.

Last year we reported that v-*erb*A causes a block in erythroid differentiation by a mechanism different from tyrosine kinases (see above). The puzzling aspect of this result was the fact that c-*erb*A, the endogenous wildtype thyroid hormone receptor (c-*erb*A), has only minor effects on erythroid differentiation. Our working hypothesis therefore was that other related nuclear hormone receptors might be involved in regulating erythroid differentiation and that v-*erb*A interferes by promiscuously repressing their transactivating function. Since thyroid hormone receptor (T3R) and retinoic acid receptor (RAR) belong to the same family of ligand-inducible transcription factors, we investigated how the activation of these endogenous receptors by ligand would influence erythroid differentiation of transformed and non-transformed erythroid cells.

In a first approach to clarify whether both receptors are expressed in erythroblasts and whether their activation would affect erythroid differentiation, we used tyrosine kinase-transformed erythroblasts. In all cases analysed so far (c-erb B/EGFR, ts-v-*erb* B, ts-v-*sea*) we found a partial induction of cell differentiation after RA treatment, followed by cell death. The criteria for differentiation were i) morphological changes (elongated cells) ii) increase of the hemoglobin content iii)

decrease of antigens specific for immature erythroblasts and increase of antigens specific for mature erythrocytes. These results suggest that retinoic acid might be able to modulate the erythroid differentiation pathway. Surprisingly, this induction could be seen in the presence of an active kinase oncogene.

Next, we analysed the effects of T3 and RA in non-transformed cells derived from normal bone marrow and embryonic blood. In bone marrow cells, a significant reduction in colony size and survival time of erythroid colonies was detected. While T3 had only weak effects, RA prevented the formation of mature colonies; the cells disintegrating as partially mature cells in the develop-ing colony The effect of RA +T3 was even more severe, suggesting a cooperative action of the two hormones.

These results only partially corresponded to the effects of RA and T3 on the expression of erythrocyte-specific genes found in these cells. Steady state RNA-levels of ALA-S and band 3 were not affected by any treatment. For the CAII gene, somewhat different results were obtained with the different cell types. In bone marrow preparations enriched for immature erythroid cells, CAII-RNA was upregulated only by T3 and no synergistic action of RA and T3 was detected. In embryonic blood cells from 10 day old embryos and in transformed erythroblasts both hormone upregulated CA II mRNA. However, the latter cells responded much more strongly to RA than to T3, a result in accordance with pilot experiments suggesting a much higher expression of RAR in the transformed erythroblasts than in the normal cell preparations. It is thus tempting to speculate that expression of the RAR is developmentally regulated in erythroid cells (since embryonic red cells respond while bone marrow cells do not) and that both receptors might interact during hematopoiesis (see below).

To analyse whether the v-*erb* A oncogene might interfere with the described effects of RA on erythroid differentiation and erythrocyte gene transcription, we analyzed the RA effect in several ts-*sea* transformed erythroblast clones containing v- and c-*erb*A or different chimaeric constructs thereof. All these cells expressed the RAR at detectable levels. As expected, v-*erb* A inhibited the T3 induction of the endogenous T3R but was unable to completely inhibit induction of differentiation and gene expression by RA. To our surprise, however, overexpressed c-*erb*A and chimaeras that contained a distinct portion of the c-*erb*A hormone-binding domain inhibited the RA-effect. These data can be interpreted by assuming that overexpressed c-*erb*A in absence of T3 can function as a repressor of retinoic acid receptor function while v-*erb*A cannot, since c-*erb*A and retinoic acid receptor might form heterodimers as shown *in vitro* by Glass et al., 1989 and others while v-*erb*A may be unable to do so. We are planning to examine this possibility in the erythroid cell system by a suitable combination of *in vivo* and *in vitro* experiments. In addition, we have started to overexpress the RAR and various RAR-T3R chimaeras in erythroblasts, using suitable retrovirus vectors to study whether, and if so under what circumstances the RAR might act as an oncogene.

Significance of gene repression by v-erb A for the leukemic phenotype: Reintroduction of the Band 3 and CA II genes into v-erb A-expressing erythroblasts.

Ch. Schroeder, I. Leitner and H. Beug in collaboration with S. Fuerstenberg and B. Vennstroem, Stockholm.

It is unclear how much the v-*erb* A-induced repression of three erythrocyte-specific genes (CAII, band 3 and dALA-S) contributes to the leukemic phenotype induced by v-*erb* A (i.e. an arrest of terminal differentiation and a profound change in growth requirements, enabling v-*erb*A erythroblasts to grow in standard media; Kahn et al,1986), since v-*erb*A could act on other, so far undetected genes. Experiments using inhibitors of Band 3 and dALA-S function in v-*erb* A-expressing erythroblasts have suggested that repression of these genes may contribute to the v-*erb* A phenotype (Zenke et al., 1988, Schmidt et al., 1986). However, the toxicity of these drugs has prevented more detailed experiments. To clearly define the role of band 3 we have therefore introduced a complete avian band 3 cDNA into a suitable retrovirus vector containing a *neo* resistance marker. When used to infect fibroblasts, high levels of the Band 3 protein were ectopically expressed at the plasma membrane of these cells. The band 3-expressing fibroblasts displayed numerous large vacuoles when grown in standard medium, these vacuoles disappeared, however, when the cells were incubated in media of more alkaline pH or treated with DIDS, a drug that inhibits the HCO3-/Cl- antiport function of the band 3 protein (Zenke et al., 1988). This indicates that the retrovirus-expressed Band 3 protein is clearly functional in cells (Fuerstenberg et al.,1990).

The band 3 gene was subsequently introduced into v-erb A-expressing erythroblasts by doubly infecting bone marrow cells with ts-v-sea-v-erb A and band 3 neo retroviruses. In contrast to ts-v-sea-v-erb A erythroblasts which did not express band 3, were arrested in differentiation and grew in standard tissue culture media, the doubly infected erythroblasts expressing both v-erb A and band 3 were unable to grow in standard media and developed large vacuoles before desintegration. In media suitable for growth of erythroblasts lacking v-erb A, however, the band 3/v-erb A cells grew with similar efficiency as v-erb A-erythroblasts and failed to form vacuoles. These results indicate that repression of the band 3 gene by v-erb A is responsible for part of the leukemic phenotype, i.e. the v-erb A-induced change in growth requirements of the leukemic cells. We are currently testing whether re-expression of band 3 also affects the differentiation arrest caused by v-erb A; pilot experiments so far argue against this possibility.

To perform similar experiments with the carbonic anhydrase gene (the activity of which is reduced but not completely blocked in v-*erb* A-containing erythroblasts) we have fused the carbonic anhydrase cDNA to viral gag sequences to allow discrimination between exogeneous and endogenous CA II protein. The gag CA-II gene was inserted into two different retrovirus vectors (containing either the *neo* gene or the ts *sea* oncogene). Introduction of these vectors into fibroblasts indicated that both viruses led to high level expression of the gag-CA II protein which exhibited an apparently unchanged enzyme activity. Initial trials to express the gag-CA II gene in erythroblasts (with or without v-*erb* A) were hampered by the apparent toxicity of the gag-CA II protein for erythroblasts, perhaps caused by drastic changes in medium requirements of these cells. These studies are being continued using different growth conditions or types of erythroid cells with the aim to determine the possible contribution of CA II suppression to the leukemic phenotype as for band 3.

II. Carcinogenesis

Differentiating mammary epithelial cell lines as tools to study oncogene function in carcinogenesis

E. Reichmann, in collaboration with T. von Rüden, IMP; A. Ullrich, Munich and G. Superti-Furga and M. Busslinger, IMP.

In our previous work (see Report 1989) an *in vitro* cell culture system was described that allowed us to study the interactions of non-tumorigenic mammary epithelial cells with cells bearing mesenchymal properties. These epithelio-mesenchymal interactions could be demonstrated to be a prerequisite for the development of differentiated structures and milk protein synthesis in mammary epithelium in culture (Reichmann et. al., 1989). Our long term goal using this system is to study whether, and if so how oncogenes may interfere with the growth and differentiation properties of both the epithelial and the mesenchymal component of the mammary gland. Furthermore we would like to investigate how epithelio-mesenchymal tissue interactions are disturbed by expressing certain oncogenes in one or the other cell type. Our present approach to this aim is to use conditional oncogenes (i.e. oncogenes that can be turned on and off) to allow comparison of the neoplastically transformed phenotype with the untransformed phenotype in cells of identical clonal origin or induction of transformation in tissue-like structures formed by these cells *in vitro*.

Two different approaches allowing the conditional expression of oncogenes have been employed by us so far. The first one was to use overexpressed plasma membrane growth factor receptors which are rendered transforming by addition of ligand. Infection of a mammary epithelial subline with a retrovirus containing the human EGF receptor (hEGF receptor) gene together with the neomycin resistance gene resulted in the establishment of cell clones which expressed the hEGF receptor to varying degrees. Surprisingly, these clones could segregate morphologically different cell types that exhibited a different response towards added EGF. In the absence of exogenous EGF, dense cultures exhibited large circular areas of polar epithelial cells expressing high levels of cytokeratins, which were surrounded by areas of nonpolar cells expressing vimentin and an ordered fibronectin network, but very little cytokeratin. When grown in the presence of EGF, unaltered epithelial cell clusters were surrounded by rapidly proliferating, spindle shaped cells expressing vimentin as well as a disorganized fibronectin network. Pilot experiments to classify this nonpolarized cell type suggest, that they may represent (dedifferentiated?) myoepithelial cells (a cell type of the mammary gland duct) since they express cytokeratin intermediate filaments together with alpha actin.

To obtain a concitional version of a nuclear oncogene (v/c-*fos*), an approach developed by Superti-Furga et. al., at the IMP has been employed. These scientists have constructed vectors expressing chimaeric c/v-*fos* – estrogen receptor proteins (c- and v-*fos* ER) generated by fusion of the mouse c-*fos* or v-*fos* protein to the hormone binding domain of the estrogen receptor. These hybrid proteins are inactive in the absense of estradiol, while the addition of the steroid efficiently activates the c- or v-*Fos* protein respectively leading to efficient transformation of fibroblasts (see Report M. Busslinger).

Using these vectors, c-*Fos* ER as well as v-*Fos* ER proteins were efficiently introduced and expressed in clonal mammary epithelial cells. In the absence of ligand, the subclones exhibited a normal polarized phenotype, as assayed by morphology, ion pumping and measurement of transepithelial resistance. The addition of either estradiol or hydroxy-tamoxifen to cell clones expressing the c-*Fos* ER, however, caused the cells to obtain a rounded morphology and destroyed their polarity according to the above criteria. Furthermore the cells synthesized casein in response to lactogenic hormones only in absence but not in presence of estrogen. The nuclei of the rounded cells were enlarged, irregularly shaped and frequently even duplicated.

We are currently analysing whether or not the "transformed", (rounded) phenotype induced by ligand-activated *fos*-ER protein in epithelial cells is reversible. Initial trials show that with-drawal of estrogen resulted in the reversion of rounded cells into a new morphological cell type which is polygon-al in shape, apparently unpolarized and expresses only moderate levels of cytokeratins.

III. Other projects

Detailed analysis of the transferrin cycle in avian erythroblasts

Peter Steinlein and Hartmut Beug in collaboration with Ernst Wagner, IMP and G. Griffiths, EMBL, Heidelberg.

To better understand processes involved in the uptake of DNA by receptor-mediated internalization using the transferrin receptor (transferrinfection-see reports of M. L. Birnstiel, E. Wagner and M. Zenke) we undertook a combined biochemical and EM-immunohistochemial investigation of the transferrin cycle in chicken erythroblasts (ts 34 AEV transformed cell line HD 3) that express high amounts of transferrin receptors (TfR) after induction of differentiation at 42°C. Biochemical analysis (pulse-chase) of the internalization and recycling of ^{125}I-labelled and fluorescent transferrin (Tf) showed that the bulk of the internalized ligand recycled rapidly (within 30 mins) while a significant fraction of ^{125}I-Tf required longer time periods (120-180 min) for recycling through a more acidic compartment (pH 4.8-5.2). No significant degradation of ^{125}I-Tf was observed within 24o min. For the complementary morphological analyses at the EM level, horseradish peroxidase (HRP) was used as a marker to identify the early and late elements of the endocytic pathway and to facilitate a stereological analysis of the compartments involved. The early structures could be sub-divided into an early endosome of complex structure and a (multivesicular body-like) endosome carrier vesicle (ECV) while the late structures were made up of the prelysosomal compartment (PLC), which has high concentrations of the cation-independent mannose-6-phosphate receptor (MPR) and the MPR-negative lysosomes, which are electron-dense vesicles. Antibodies against Tf and TfR were used on cryo-sections to determine the ultrastructural localization of these molecules with respect to the compartments of the endocytic pathway. As expected, there was significant TfR labelling on the cell surface. The bulk of intracellular Tf/TfR was found in the early endosome (5 min) compartment. The ECV and the PLC gave labelling densities for Tf that were 40% and 11% of that found over the early endosome. No significant labeling for Tf and TfR was found associated with the Golgi complex at steady state. Finally, since no label for Tf/TfR was found over lysosomes these data provide additional evidence that a significant fraction of the Tf TfR complex recycles via the PLC.

Developmental regulation of three members of the avian erythroid transcription factor multigene family, NF-E1

H. Beug, in collaboration with Doug Engel, Chicago.

Recently, the laboratory of D. Engel identified three avian c-DNA's highly homologous to mammalian NF-E1, an erythroid transcription factor regulating expression of several erythrocyte specific genes. All three factors are highly homologous to each other and recognized NF-E1 consensus sequences in specific DNA-binding- and transient transfection experiments. The three proteins, however, differ in their tissue specificiy; while NF-E1a was eclusively expressed in erythroid cells, NF-E1b was expressed in a few additional tissues (Brain, liver, muscle). NF-E1c was expressed in erythroid cells, brain and (very extensively) in lymphoid leukemic cells. None of the factors was expressed in myelomonocytic cells. Most interestingly, the three factors were differentially expressed during erythroid differentiation: NF-E1a was present in all stages, NF-E1b was downregulated during differentiation and absent in mature cells, while NF-E1c was not expressed in immature, leukemic erythroblasts and upregulated during erythrocyte maturation. We are currently analysing expression of these NF-E1 genes in erythroid cells containing c- or v-*erb* A to study whether, and if so how any of them are regulated by *erb* A.

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DNA Methylation and Genome Function

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Introduction

Adrian Bird

Our interest in the function of DNA methylation in animals has led us to study two extremes of chromatin structure. On one hand CpG islands, which are non-methylated, represent "open" or "active" chromatin, as would be expected of sequences that surround the promoters of most genes. On the other hand, bulk vertebrate DNA is methylated and transcriptionally inert. In the past year we have concentrated on the relationship between CpG methylation and transcriptional inertia. At the cellular level we were surprised to find that more than half of the CpG islands in permanent cell lines are in a methylated state (Antequera), the implication being that most of the genes in these cells were stably repressed by methylation. CpGs in the methylated islands were nuclease-resistant in nuclei, raising the possibility that methyl-CpG binding proteins (MeCPs) are bound at these sites. Two MeCPs (1 and 2) have been characterised and MeCP-2 has been purified (Meehan and Lewis). In order to determine whether MeCPs could be reponsible for methylation-mediated repression of transcription, methylated and nonmethylated promoters have been tested in nuclear extracts and transient transfection assays (Boyes). The results strongly suggest that MeCP-1 is an important mediator of transcriptional repression *in vivo* and *in vitro*.

The finding that many genes in cell lines are inactivated by CpG island methylation prompted us to ask whether inactivation of recessive oncogenes in this way could promote aberrant growth in culture (Ulrich). In collaboration with Hartmut Beug, it has been shown that p53 expression is disrupted in immortal chicken cell lines, but not in their mortal progenitors. There is no correlation between the presence or absence of p53 and either the transformed state of the cells or their ability to differentiate.

Finally, we have pursued a long-standing interest in the evolutionary origins of the CpG island phenomenon (Cross). Several species of fish have non-methylated islands, but unlike mammals and birds, these islands are not GC-rich. The finding raises the possibility that the GC-richness of mammalian CpG islands is not critical for their function, but is the inevitable consequence of DNA repair in regions of "open" chromatin. GC-rich islands, like GC-rich isochores, could be caused by an imbalance of dNTP pools that occurs in birds and mammals, but not in fish.

Methylated DNA binding proteins

Richard R. Meehan, Joe D. Lewis

The dinucleotide CpG is the major site of DNA methylation (at the 5 position of cytosine) in vertebrates. A consistent observation is that methylated DNA is associated with gene inactivation and the formation of inactive chromatin. On a mechanistic level this suggests the presence of factors in the nucleus which can distinguish between methylated and non-methylated DNA. We have used different assay methods to identify two nuclear proteins which bind preferentially to methylated DNA. These proteins are termed "methylated CpG binding proteins" or MeCP's. MeCP-1 (Mr 120 kD) was identified by the electrophoretic mobility shift assay, and MeCP-2 (Mr 84 kD) by the South-Western assay. Both proteins were detectable in mouse and rat nuclear extracts. Neither of the MeCPs has a specific sequence requirement other than the presence of a number of symmetrically methylated CpGs in the test molecule. Despite the similarity in DNA binding characateristics between MeCP-1 and MeCP-2 we can biochemically distinguish the two activities by a number of criteria. To date MeCP-1 has proven refractory to purification although progress has recently been made (S. Cross). MeCP-2, on the other hand, has been purified to virtual homogeneity from a number of rat and pig tissues. Antibodies have been raised in rabbits to purified rat MeCP-2 and Western blots with anti-MeCP-2 antibodies detect a single band of Mr 84kD in rat nuclear preparations. These antibodies are currently been used to screen expression libaries for potential MeCP-2 cDNAs. We do not as yet have a biological assay for MeCP-2 activity, but our data suggests that it is intimately associated with chromatin, and may therefore be involved in the nuclease-resistance of methylated CpGs in the nucleus. Future experiments will attempt to test this possibility.

Mechanism of Inhibition of Transcription by DNA Methylation

Joan Boyes

A variety of studies have established that methylation of CpGs near the 5' end of a gene leads to transcriptional repression. Methylation may bring about this inhibition by either of two mechanisms: a *direct* mechanism whereby transcription factors see a methyl-CpG as a mutation in their binding site and thus are unable to bind; or an *in- direct* mechanism whereby nuclear component(s) associate with methylated DNA and prevent transcription factors from binding. To distinguish these two mechanisms, four different promoters (human alpha globin, Herpes Simplex virus thymidine kinase, mouse X-linked phosphoglycerate kinase, and a retroviral LTR) were methylated to completion and studied in both transient transfection experiments and in *in vitro* transcription extracts. All the constructs were repressed by CpG methylation in transient transfection assays. In nuclear extracts inhibition of transcription was observed at low concentrations of the methylated template, but not at high template concentrations suggesting that the inhibition of transcription by methylation was indirect via a component which is limiting in the extracts. In agreement with this, addition of any meth-ylated competitor DNA to the *in vitro* transcription assays overcame the inhibition. Two proteins have been identified previously which bind to methylated DNA in a sequence non-specific manner (MeCPs -1 and -2) and provided obvious candidates for the indirect inhibitor. By making use of substrates that can distinguish MeCP-1 and -2, it was shown that the binding properties of the inhibitor were indistinguishable from those of MeCP-1.

Is MeCP-1 also involved in the inhibition of transcription by methylation in living cells? This was investigated in three ways. Firstly, constructs with increasing affinities for MeCP-1 were transiently transfected into HeLa cells. The inhibition of transcription matched exactly the affinities of the templates for MeCP-1. A second experiment showed that cotransfection of methylated, but not non-methylated, competitor DNA could go some way to alleviating the inhibition of transcription from a meth-ylated template *in vivo*. Finally, transient transfection of methylated and non-methylated templates into a cell line with very low levels of MeCP-1 (F9 cells) showed that the inhibition of transcription by methylation was also much reduced. Taken together, these results strongly suggest that MeCP-1 is involved in the indirect inhibition of transcription *in vivo* by DNA methylation.

Blockage of p53 transcription during immortalisation of chicken cell lines.

Eugen Ulrich; collaboration with Hartmut Beug

We have used retrovirus-transformed chicken cells to investigate the expression of the tumour suppressor genes retinoblastoma (Rb) and p53 during the process of cell immortalisation. In the chicken system, clones of transformed cells are initially mortal since they stop dividing after 30-50 generations and enter a prolonged crisis phase. After several months of crisis a few dividing cells can be detected. These cells can be grown out and will eventually divide indefinitely in culture; that is, they are immortal. Hartmut Beug has followed several cell clones from different chicken tissues through this progression, and has frozen cells at various stages. These cells therefore provide a unique resource for the study of the genetic changes that accompany cell immortalisation. We initially became interested in the system because many genes in cultured cell lines are known to be inactivated by DNA methylation at CpG islands (see Antequera), and because inactivation or alteration of p53 and/or Rb genes have frequently been observed in mammalian transformed cells and tumours. Combining these points, we wished to find cells that no longer expressed the recessive oncogenes, and then ask whether the genes were inactivated by CpG island methylation. So far we have completed only the first stage of the analysis, but the results are already of interest.

When we looked at the mRNA levels of Rb and p53 we found that Rb is expressed at apparently normal levels in all mortal and immortal cells investigated so far. On the other hand p53 expression showed dramatic differences between mortal and immortal cell lines. All mortal clones showed normal levels of p53 mRNA, but in immortal cell lines derived from these clones 6 out of 8 showed no detectable p53 RNA, and one showed a shortened transcript. Line tsAEV34A6L1 was the only immortal line to have apparently normal p53 mRNA. With this one exception, the correlation between abnormal p53 expression and immortalisation was therefore strong. In contrast there was no obvious correlation between abnormal p53 expression and cell transformation, or with the ability to terminally differentiate. An extreme model is therefore that inactivation or modification of p53 is an essential prerequisite for the transition of mortal to immortal cell lines in chicken. This model makes the strong prediction that the apparently normal p53 RNA in line ts34A6L1 will contain mutations that alter p53 function. We are currently testing this prediction.

De novo methylation of CpG islands in permanent cell lines

Francisco Antequera, Joan Boyes

CpG islands are normally non-methylated in cells of the animal, even when the associated gene is transcriptionally silent. It has been reported, however, that repression of some genes in permanent cell lines correlates with de novo methylation of their CpG islands. We were interested in determining to which extent this phenomenon occurs in mouse and human cell lines. Quantitative analysis showed that its incidence is much higher than previously understood, amounting in some cases to more than half of the total number of CpG islands per nucleus. *De novo* methylated CpG islands were found in several island-associated tissue-specific genes whose expression is most likely non-essential in culture. Conversely, housekeeping genes that are probably required for survival were never found methylated in any cell line. *De novo* methylated CpG islands adopt an Msp I-inaccessible conformation in the nucleus compared to their non-methylated counterparts. These results suggest that *de novo* methylation of CpG islands is widespread in permanent cell lines and could account for the loss of specific functions in cultured cells.

Non-methylated islands in fish genomes are GC-poor

Sally H. Cross

In most vertebrates the CpG island fraction of genomic DNA can be detected by end-labelling after digestion with methylation-sensitive restriction enzymes. Surprisingly when this was done with DNA from some fish species there was no release of an obvious CpG island fraction. Analysis of housekeeping genes in two fish species showed that the 5' ends of the genes are island-like in that they were non-methylated and contained CpG at the expected frequency, but they differed from islands in birds and mammals in that their GC-content was not elevated compared to bulk genomic DNA. In this they resembled invertebrate genes which are found in methylation-free domains having the same GC-content as the rest of the genome. The presence of GC-poor islands explains why the island fraction was not readily detected in the end-labelling experiment. Clearly the CpG island phenomenon does not depend on high local GC-content in all vertebrates.

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Transcription factors involved in signal transduction and differentiation

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I. The role of the oncoprotein Fos in signal transduction

Introduction

The proto-oncogene c-fos has been implicated in the control of proliferation and differentiation and its protein product is an essential component of the transcription factor AP-1. The Fos protein is thought to act as a central regulator in the transduction of short term stimuli into long term alterations of the gene expression programme. The important target genes and hence the molecular mechanism of Fos action are, however, still unknown mainly due to the lack of conditional fos expression systems. During the last year we have established a novel and tightly regulated Fos induction system which will allow us to study the biological role of Fos by identifying Fos target genes.

Fos-estrogen receptor fusion proteins as a tool to study Fos action and to isolate Fos-responsive genes

Giulio Superti-Furga, Gabriele Bergers, Sylvia Braselmann, Paula Graninger

Picard et al. (1988) have shown that the activity of certain proteins can be brought under hormonal control by fusing them to the ligand-binding domain of steroid receptors. In last year's report we have shown that chimaeric molecules consisting of either c-Fos or v-Fos fused to the hormone-binding domain of the rat glucocorticoid (Fos-GR) or human estrogen receptor (Fos-ER) can activate AP1-depedent transcription in a strictly hormone-dependent manner. In the meantime we have derived several rat and mouse fibroblast cell lines that constitutively express the Fos-ER fusion protein. Upon addition of hormone, such cell lines undergo morphological transformation which is first visible after 24 hours and which is completed in a few days. Moreover, these cells are able to form foci and grow in soft agar only in the presence of hormone. Transformation by Fos-ER is completely reversible, as cells which have been incubated with hormone for more than two weeks return to normal morphology within 48 hours, if reseeded in hormone-free medium. This indicates that the Fos-ER induction system is tightly controlled and that continuous Fos activity is required for maintaining the transformed phenotype. Besides its ability to activate AP1-dependent transcription, Fos is able to repress the serum-induced activity of several immediate-early gene promoters including its own. We have found that treatment of our Fos-ER cell lines with hormone causes a fivefold repression of serum-induced *fos* mRNA synthesis similar to that of a highly transformed control cell line which constitutively expresses the c-Fos protein. We have used this repression assay to test the kinetics of activation of the Fos-ER fusion protein. Addition of hormone only seconds before serum stimulation is sufficient to cause repression to an extent which is identical to that obtained by 24 hour pretreatment with estrogen. By performing the repression assay in the presence of cycloheximide, we could prove that Fos is able to directly repress its own promoter without the need for ongoing protein synthesis.

Our main goal is to identify Fos-regulated genes which are involved in the control of cell proliferation. For this purpose we have constructed a subtracted cDNA library in lgt10 vectors using RNA from hormone-induced versus uninduced Fos-ER expressing rat fibroblasts. We

have differentially screened this library with radio-labelled cDNA probes derived from RNA of either induced or uninduced cells. A first round of differential screening led to the identification of several cDNA clones corresponding to abundant Fos-regulated mRNAs. These mRNAs accumulate rapidly within the first hours after hormone addition which has been shown to be due to immediate transcriptional activation by the Fos-ER protein. We are currently determining the nucleotide sequence of these cDNA clones which may provide a hint as to the function of the encoded proteins. To facilitate the isolation of less abundant cDNA clones we are now using subtracted cDNA probes for a second round of screening. These probes are enriched for Fos-induced transcripts by subtraction of the sequences common to both estrogen-treated and untreated cells.

Having established the conditions for cloning Fos-responsive genes we expect to isolate many more members of this gene family in the near future. Sequence analysis may provide some information about the nature of the proteins encoded by these genes. We expect to identify some structural proteins, which may be important effector molecules responsible for the change in cell shape that is observed upon transformation. Other proteins may be isoforms of enzymes that are required for some of the metabolic changes found in cancer cells. We might also identify regulatory proteins, such as protein kinases or transcription factors. In order to specifically search for cDNA clones encoding proteins of this latter class it might be worthwile to explore an alternative to the differential screening approach which relies on PCR amplification of cDNA sequences from our subtracted library by using degenerate oligonucleotides corresponding to highly conserved protein domains. Our ultimate goal is to identify those members among the Fos-responsive genes that are sufficient to elicit cell transformation when overexpressed either alone or in combination.

Morphological and molecular effects of regulated c-fos expression in PC12 cells

Chris Wrighton

A large body of evidence suggests that Fos may play a key role in both the functioning and development of nervous tissue. Fos protein rapidly appears in the central nervous system after stimulation and can be detected in developing nervous tissue in sections of mouse embryos. In the latter instance it is conceivable that Fos plays a role in tissue maturation by activating key genes. With the long term goal of isolating neuron-specific Fos-regulated genes we have generated, by retroviral-mediated gene transfer, PC12 cell lines in which Fos activity can be tightly and specifically regulated. This was facilitated by the development of a Fos-estrogen receptor fusion protein possessing Fos activity only in the presence of hormone (see above). In the absence of estrogen, Fos-ER expressing cells are indistinguishable from the uninfected parental cells. Hormone addition rapidly alters cell morphology resulting in a flattened, pseudo-syncitial appearance. This effect is reversible, though over a 2-3 week period. Cells treated for 24 hours with estrogen fail to subsequently differentiate to sympathetic neuron-like cells in response to NGF treatment. This block of morphological differentiation is mirrored at the molecular level in the inhibition of expression of neuronal genes. There is no critical time period during which Fos exerts this effect. Activation of Fos-ER in extensively differentiated cells results in a rapid dedifferentiation and resumption of cell division. Moreover, cells "primed" with NGF for a week do not extend processes when plated on a substrate in the presence of hormone.

All these effects are Fos-, rather than ER-specific. Control cell lines constitutively expressing native c-Fos exhibit an identical morphology to estrogen treated Fos-ER cells and also fail to differentiate upon NGF treatment. Furthermore these effects apparently require that the Fos-ER protein binds DNA since they are not generated by a non-DNA binding mutant protein containing a proline insertion in the DNA-binding domain (M. Nicklin, unpublished).

Our immediate aim is to analyse the expression of a wider range of neuronal markers in Fos-ER expressing, NGF-treated cells. This will reveal whether Fos prevents induction of neuron-specific genes in general or interferes with the expression of only a few of these genes. A general effect would strongly argue that Fos represses the activity of a neuronal "master regulator". The main goal shall be to use subtractive cDNA cloning technology to isolate such a hypothetical "master regulator" and other neuron-specific Fos-regulated genes that underly the morphological phenomena so far characterised.

II. Characterization of the B-cell-specific transcription factor BSAP

Introduction

The B-cell-specific transcription factor BSAP was identified as a mammalian homologue of the sea urchin protein TSAP which is responsible for transcriptional regulation of four tissue-specific histone genes by binding to their promoters (Barberis et al., 1989). Protein-DNA binding studies showed that the mammalian BSAP protein binds to these four recognition sequences in an identical manner to the sea urchin factor. BSAP is exclusively expressed during B-cell differentiation from the early pro-B cell to the mature B-cell stage. BSAP activity is, however, absent in the terminally differentiated plasma cell as well as in all other cell types tested. Competition experiments indicated that BSAP does not bind to known regulatory regions of immunoglobulin and class II major histocompatibility genes. BSAP was, however, shown to be a B-cell-specific transcription factor, as its recognition sequence is sufficient as an upstream promoter element to bring about transcription of a test gene in B-cells, but not in other cell lines (Barberis et al., 1990).

The human CD19 gene is a potential BSAP target gene.

Zbynek Kozmik, Petra Dörfler, Ben Adams, Shirley Wang

CD19 is a cell surface protein of B-lymphocytes that is involved in signal transduction and that is expressed throughout differentiation up to the mature B-cell stage. CD19 is, however, absent from immunoglobulin-secreting plasma cells. The similarity in the expression pattern of BSAP and the CD19 gene suggests that BSAP may regulate the CD19 gene. To investigate this hypothesis we have studied the expression of the CD19 gene in 21 different lymphoid cell lines by S1 nuclease mapping. This analysis revealed complete correlation between BSAP and CD19 gene expression. As a next step, the chromosomal CD19 gene was isolated from a human cosmid library using a cloned CD19 cDNA probe. DNA fragments containing BSAP-binding sites were identified by multiple band-shift assay (Kozmik and Paces, 1990) using a highly purified BSAP fraction (see below). At least four high affinity binding sites were identified in two introns close to the 5' end of the gene. The contribution of these recognition sequences to the B-cell specific transcription of this gene is being investigated by in vitro mutagenesis followed by cell transfection experiments.

Biochemical purification of BSAP

Ben Adams

BSAP consists of a single polypeptide with an apparent molecular weight of ~50 kD. We have purified this polypeptide in a two-step procedure from nuclear extracts of the human B-cell line BJA-B. This results in a 10'000-fold enrichment of BSAP with the final protein fraction being 5-10% pure. The BSAP polypeptide was ultimately purified by gel electrophoresis and is currently being sequenced. Peptide sequence data should enable the cloning of BSAP cDNA and its gene. The cloned gene will open the way for a structure/function analysis of BSAP and for studying the role of BSAP in B-cell differentiation by using retroviral-mediated gene transfer into hematopoietic stem cells. A biochemical and functional analysis of the BSAP promoter should allow us to address the interesting question what regulates the regulator in early B-cell development.

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Cell Cycle Conrol in Yeast

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The function of SWI4 and SWI6 in the entry of yeast cells into the cell cycle.

Kim Nasmyth, Leon Dirick, Rita Taba, and Shanthini Sockanathan.

In the yeast Saccharomyces cerevisiae, the decision to undergo cell division is made during G1 at a point called START. Haploid yeast cells in early G1 have three possible developmental fates: 1) Entry into a quiescent state known as stationary phase if there are insufficient nutrients in the medium. 2) Differentiation into gametes capable of conjugation, a process which is induced by pheromones secreted by cells of the opposite mating type. 3) Entry into the mitotic cycle by initiating DNA replication. Once a cell reaches a certain stage in late G1 called START, however, it becomes commmitted to the last of these options, mitosis, and will complete cell division even if starved of essential nutrients and even in the presence of sex pheromones. Genetic analyses have identified two types of proteins required for START: a protein kinase encoded by the *CDC28* gene and a set of proteins with limited but significant homology to mitotic cyclins (see U. Surana's abstract) which are encoded by *CLN1, 2,* and *3*. *CDC28* is an essential gene but *CLN1, 2, 3* have redundant functions; any one of these three genes is sufficient for viability. It is presently thought that the CLN proteins associate with the CDC28 catalytic subunit to form an active kinase which is capable of phosphorylating (and thereby activating?) proteins involved in the initiation of DNA replication.

We are interested in the control of START: why it only takes place once cells reach a certain cell size and how it is repressed by pheromones. The observation that it is possible to isolate mutations in *CLN* genes (but so far not in *CDC28* itself) which cause START to take place at a smaller cell size than normal or in the presence of pheromones suggests that it is normally regulated by the activity of the CLN proteins rather than the state of the CDC28 catalytic subunit. What then regulates the activity of the *CLN* genes?

The SWI4 and SWI6 genes were originally identified as regulators of the HO endonuclease gene which initiates mating type switching. They bind to a repeated motif in the *HO* promoter (called CACGA4) which causes *HO* to be activated only transiently during the cell cycle as cells undergo START. Neither *SWI4* nor *SWI6* are essential genes but double mutants are lethal, suggesting that together they fulfill an essential function for the cell. Our analysis of the phenotypes of various *swi*, *cln* mutant combinations suggests that *SWI4* is required for the full activity of *CLN1* and *CLN2* whereas *SWI6* is required for the activity of *CLN1* and *CLN3*. Consistent with this conclusion is our observation that the lethality of *swi4 swi6* double mutants can be suppressed by transformation with a high copy number plasmid containing the *CLN2* gene. Measurements of *CLN1, 2, 3* RNA levels in *swi4* and *swi6* mutants suggest that both *SWI4* and *SWI6* exert their effect on *CLN1* and *CLN2* at the level of transcription but that *SWI6* is surprisingly not required for *CLN3* transcription. We are therefore currently studying the role of *SWI4* and *SWI6* in the regulation of START.

The role of the CDC28 protein kinase in regulating the entry of the HO transcription factor SWI5 into the nucleus

Thomas Moll, Graham Tebb and Kim Nasmyth

The yeast HO gene encodes a site specific endonuclease which initiates mating type switching in haploid homothallic S. cerevisiae strains. Activation of the HO endonuclease is dependent on at least six different genes, SWI1-6. One of these genes, SWI5, codes for a site specific DNA-binding protein. This protein is expressed in a cell cycle dependent manner in S, G2 and M phases, during which it accumulates in the cytoplasm. Upon entry of cells into G1, the SWI5 protein is translocated into the nucleus.

Deletion analysis on the protein has identified a short portion of SWI5 which is both necessary and sufficient to translocate the protein into the nucleus in a cell cycle dependent manner. In addition to a positively charged karyophilic sequence this domain contains three consensus sequences potentially recognized by the cdc2/CDC28 protein kinase. Destruction of any of these kinase sites by site-directed mutagenesis results in partial deregulation of subcellular localization and leads to premature entry of the SWI5 protein into the nucleus. When all three sites are mutated, the protein enters the nucleus constitutively as monitored by both in situ indirect immunofluorescence and activation of a SWI5 dependent reporter gene. Biochemical analysis has shown that these sites are phosphorylated by purified CDC28 kinase in vitro and that they are phosphorylated in a cell cycle dependent manner in vivo. In vivo phosphorylation or dephosphorylation of these residues correlates with either cytoplasmic or nuclear localization of the protein respectively. SWI5 from cells carrying a ts-allele of CDC28, which is defective in kinase activity at the non-permissive temperature, is not phosphorylated at these residues and is located in the nucleus in cells arrested at the restrictive temperature.

The cumulative evidence thus suggests that SWI5 is phosphorylated by CDC28 kinase activity during most of the cell cycle, thereby causing its retention in the cytoplasm. Upon destruction of the kinase activity at the end of mitosis, dephosphorylation of the SWI5 protein would subsequently lead to its translocation to the nuclear compartment.

A new role for MCM1: MCM1 binding to the SWI5 Promoter is Required for Cell Cycle Regulated Transcription of the SWI5 Gene.

David Lydall, Gustav Ammerer and Kim Nasmyth

Yeast cells switch mating type after a gene conversion event is initiated by the *HO* endonuclease at the *MAT* locus. Mating type switching is regulated; mother cells switch and produce two progeny of opposite mating type, whereas daughter cells do not switch. The regulation of mating type switching is achieved primarily by the control of *HO* expression. *SWI5* encodes a transcription factor which is required for *HO* gene expression; it also plays an essential role in restricting *HO* transcription to mother cells. *SWI5's* role as the determinant of mother cell specific mating type switching requires that it is not expressed during G1; *SWI5 's* transcription is usually activated in S phase and repressed as cells enter G1 of the next cell cycle. Artificially induced expression of *SWI5* in G1 populations causes an aberrant pattern of mating type switching in which daughter cells now switch mating type. The pattern of *SWI5* expression is unlike any other cell cycle regulated gene in yeast and we would like to understand how events in the cell cycle control its transcription.

Analysis of the SWI5 gene has allowed us to identify the sequences within it which control its expression. A small 60bp sequence within the promoter is both necessary for transcription of SWI5 and sufficient to impose cell cycle regulated transcription on a reporter gene. There are binding sites for at least two proteins within the SWI5 UAS; one binding site is for the known transcription factor MCM1, the other is for another as yet unidentified protein. Mutations in either protein binding site cause a drastic reduction in promoter function and we conclude therefore that efficient transcription requires the production of a ternary complex comprising MCM1, another protein (FATF, SWI5 Activating Transcription Factor) and the SWI5 UAS. MCM1 has previously been shown to have a central role in the transcription of genes involved in the control of yeast cell mating type, and a role in the maintenance of plasmids within yeast cells. The MCM1 protein contains a recently identified DNA binding motif which is also present in the human SRF protein (serum response factor) and in the proteins encoded by the plant homeotic genes agamous and deficiens. Evidence for the existence of a protein (FATF) which can interact with MCM1 on the SWI5 promoter comes from the observation that fractionated yeast extracts contain an activity which will bind to the SWI5 UAS only when exogenous MCM1 is added.

As described above there are mutations in the MCM1 binding site which destroy UAS activity, there are also mutations in the MCM1 binding site with minimal effects on transcription. One might expect that mutant DNAs with the highest affinities for transcription factors would be those which activate transcription most efficiently. However this is clearly not always the case. We have identified a mutant SWI5 UAS which has a much reduced affinity for MCM1 in vitro, yet which can support near wild-type levels of transcription in vivo; in contrast there are other mutant UASs with less drastically reduced affinities for MCM1 which are not able to support transcription. Our interpretation of these results, consistent with a recent report from Tan and Richmond (Cell (1990) 62 p367), is that the conformation of MCM1 on DNA is influenced by the site to which it binds. Only when bound in the appropriate conformation is MCM1 capable of interacting with FATF on the SWI5 UAS to activate transcription. In other words:

"It's not what you do, it's the way that you do it."

The regulation of mitosis in Saccharomyces cerevisiae: a role for CDC28 kinase and cyclin

U. Surana, H. Robitsch, A. Amon and K. Nasmyth

The budding yeast *Saccharomyces cerevisiae* regulates progression through the cell cycle in response to external stimuli such as starvation and the presence of pheromones. The controls exerted by these stimuli predominantly operate at the G1/S boundary eliciting a variety of cellular responses including a delay in the commitment to mitosis (START). Passage through START requires CDC28 kinase and proteins that bind to it, the so-called G1 cyclins. In organisms such as the fission yeast, frog and starfish where G2/M transition is the main mode of cell cycle control, a homologous kinase, cdc2, in association with a different type of cyclin (G2 cyclin) regulates the entry into mitosis. It is remarkable that the Cdc28 is conserved in organisms evolutionarily as distant as yeast, frog and man suggesting that some of the cell cycle controls involving this kinase and cyclins may also be conserved. However, G1-specific cyclins are yet to be found in these organisms; likewise, in S. *cerevisiae*, no G2 cyclins have so far been reported.

Since most conditional alleles of *CDC28* cause G1 arrest, it has been thought that the *CDC28* function may not be needed during the transition from G2 to M phase and that the budding yeast perhaps lacks the G2/M control. This notion is consistent with the discovery so far of only G1-specific cyclins in this organism. However, the behaviour of an exceptional allele, *cdc28-1N*, suggests that *CDC28* like its counterpart $cdc2^{+}$ in *Schizosaccharomyces pombe*, may also be required for passage through mitosis. *cdc28-1N* cells, though normal with regard to G1 function (START), arrest in G2 (or M). We have characterized this allele further and find that whereas a START-defective allele (*cdc28-4*) exhibits no histone H1 kinase activity at the restrictive temperature, the activity of cdc28-1N remains high. This indicates that the failure of *cdc28-1N* cells to complete mitosis is not simply due to a lack of histone H1 kinase activity. In order to understand why the cdc28-1N allele causes mitotic arrest, we have isolated seven genes which in high copy number can suppress the growth defect of cdc28-1N mutants. Two of these genes (*CLB1 and CLB2*) encode proteins which bear strong homology to B-type cyclins (G2 cyclins). Disruption of either gene does not affect progression through the cell cycle but a simultaneous disruption of both is lethal. Moreover, whereas a deficiency of either Clb1 or Clb2 in combination with *cdc28-4* has no detrimental effect on growth at the permissive temperature, the *cdc28-1N*/*clb2*::LEU2 double mutant is nonviable.

We propose, therefore, that the mitotic defect caused by the cdc28-1N allele is due to its inability to interact with the B-type cyclins (G2 cyclins). The results are consistent with the idea that CDC28 function is needed at two stages in the cell cycle, once at the G1/S boundary and again during mitosis. Whereas the START function requires association with G1 cyclins, the role in mitosis involves interaction with the CLB gene products (G2 cyclins). The discovery of G2 cyclins in the budding yeast makes this the first organism in which both G1 and G2 cyclins have been shown to drive the mitotic cycle.

A successful progression through M-phase requires not only the correct initiation but also a timely termination of the mitotic processes. It is already known that in the fission yeast and frog oocyte metaphase to anaphase transition leads to a catastrophic loss of the cdc2 kinase activity. To further our understanding of mitosis in the budding yeast we asked what is the state of the CDC28 kinase activity at the time of exit from M phase. In order to address this question, we have measured *CDC28*-dependent histone H1 kinase activity in cdc15 mutants. These mutants arrest in anaphase at the nonpermissive temperature and fail to exit mitosis due to their inability to complete late nuclear division. We find that although the *cdc15* cells exhibit substantial H1 kinase activity at 37°C, this activity rapidly declines as cells are released from the anaphase-arrest. The loss of the kinase activity is concomitant with a complete breakdown of the internuclear spindles, a hallmark of cells exiting mitosis. These observations suggest that in the budding yeast completion of mitosis requires **CDC15** function and is accompanied by a sudden loss of histone H1 kinase activity. Cloning and sequencing of **CDC15** gene has revealed that it encodes a serine/threonine protein kinase of 107 kd molecular weight. It is conceivable that one of the functions of **CDC15** gene product is to actively participate in the destruction of CDC28 kinase activity by phosphorylating the components of M phase-active kinase complex. The studies are under way to determine the interaction between **CDC15** and **CLB** gene products.

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Gene-Function in Mammalian Development and Differentiation

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Our research programme continues to focus on the function of genes in stem cell differentiation and development. 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 recombinant genes into the germ line of mice by microinjection of DNA into fertilized eggs. (ii) A complementary approach uses embryonic stem (ES) cells as target cells for gene transfer to ectopically express 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 gene function during hematopoietic development we are using retroviral vectors to express recombinant genes in murine bone marrow cells.

The specific goals of these approaches are to analyze the function of growth control genes in mammalian differentiation and development, to study the molecular events leading to tumour formation and to generate mouse models for human genetic diseases.

I. Effects of c-fos on bone and cartilage differentiation in transgenic and chimaeric mice

Z.-Q. Wang, U. Möhle-Steinlein, A. Grigoriadis, and K. Schellander

We have used transgenic mice and embryonic stem (ES) cells to analyze the role of c-*fos* during development and skeletal cell differentiation. Transgenic mice were generated by microinjection of a construct carrying c-*fos* under the control of the murine H2-K^b class I MHC promoter and having the 3' non-coding part replaced by the FBJ LTR (H2-c-*fos*LTR). Four independent transgenic founder mice were generated, one of which developed specific lesions in almost all bones of the body as early as 4 weeks of age. The lesions progressed to fully malignant osteosarcomas in older animals. This transgenic line was bred to homozygosity and investigated more closely.

Histological analysis revealed that the tumors which arise with 100% penetrance contain large areas of neoplastic bone and many areas of fully differentiated cartilage. RNA analysis in adult animals showed that the transgene was expressed in several different tissues including bones and tumors. Thus, despite expression in other organs, specific phenotypic alterations only occurred in bone tissue. Expression studies during early development indicated that whereas the transgene was not expressed before birth, post-natal expression of exogenous c-*fos* followed a distinct tissue-specific pattern. Expression in the lungs and heart started within the first week of age while

the onset of expression in bone tissue (calvaria, long bones, spine) occurred at approximately 2-3 weeks of age, prior to the onset of tumor formation (4-6 weeks).

To investigate further the consequences and possible function of c-fos during early development we have also introduced into ES cells a construct containing genomic c-fos under the control of the human metallothionine promoter (MT-c-fosLTR). Ten clones were selected which express different levels of exogenous c-*fos* and 5 were used to generate chimaeric mice after introduction into blastocysts. No effect was observed during early development, however, as early as 3-4 weeks after birth a high frequency of cartilage tumours developed in chimaeras (Fig.1A). The tumours appeared to originate in the leg joints, spinal cord, ribs and throat, and were highly invasive. Histological examination of the tumours revealed areas containing differentiated chondrocytes and some bone (Fig.1B). In addition, large ectopic foci of differentiated chondrocytes were present in the bone marrow and in the cortical bone. Despite high levels of exogenous c-*fos* expression and contribution by the ES cells to several tissues of these mice, histological abnormalities were only observed in cartilage and bone tissues. The results obtained in both transgenic and ES cell chimaera studies suggest that overexpression of c-*fos* specifically interferes with normal cartilage and bone development.

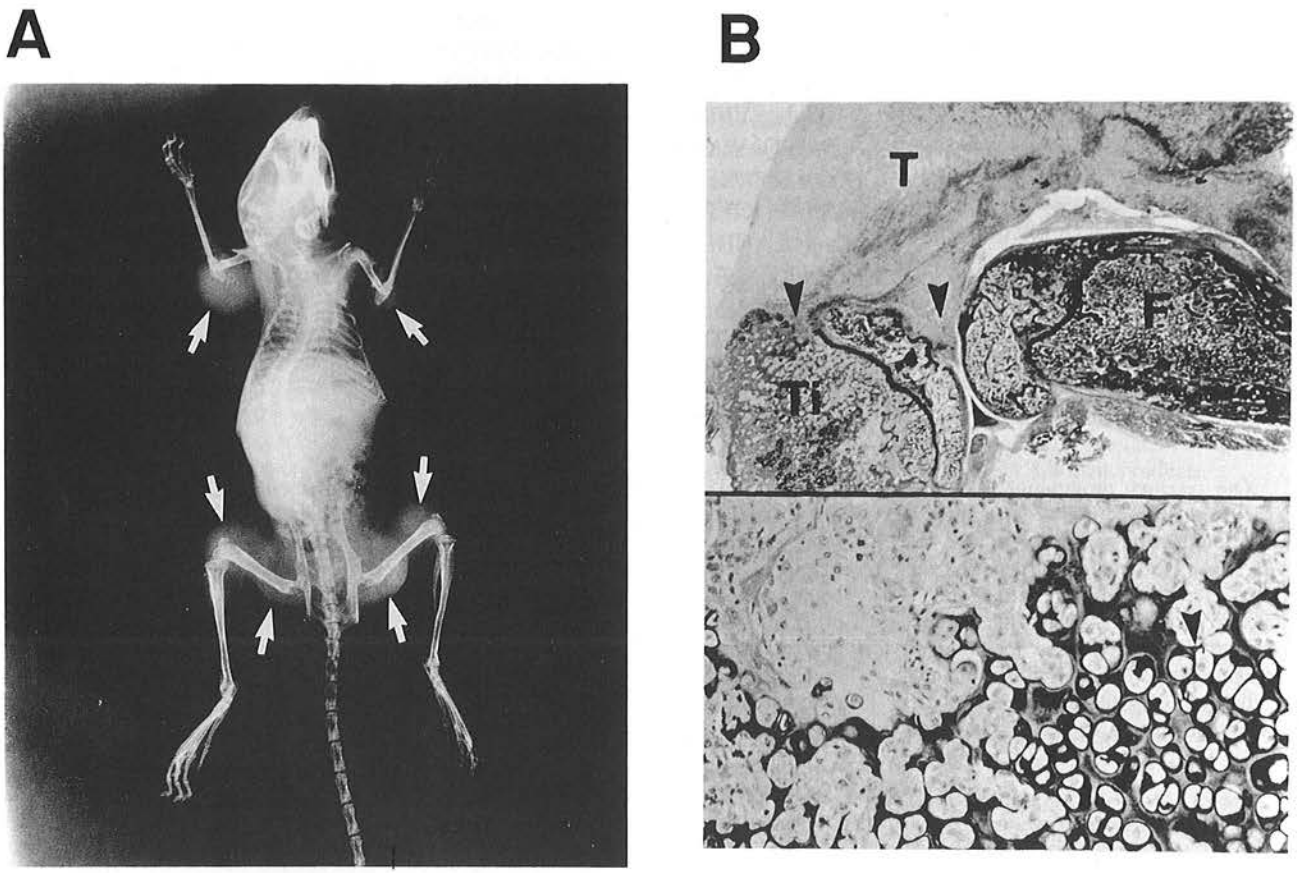

Figure 1. Consequences of c-fos overexpression in ES-cell chimaeras.

A. X-ray radiograph of a 6-week old MT-c-*fos*LTR chimaera. Large sarcomas develop in the joints of the long bones (arrows) with no obvious effects on the skeletal bones. **B**. Pathology of a typical MT-c-*fos*LTR chimaeric mouse. Upper panel: histological section through the knee joint of a chimaera. The femur (**F**) and tibia (**Ti**) are completely surrounded by a large tumour (**T**). The tumour can be seen infiltrating into the bone and joint (arrowheads). Lower panel: higher magnification of a tumour showing areas of differentiated chondrocytes (arrowhead). Paraffin sections are 4-6 mm thick and are stained with haematoxylin and eosin.

II. c-*jun* expression in ES cells and transgenic mice

F. Hilberg and K. Schellander

The nuclear proto-oncogene c-*jun* codes for an AP-1 binding activity and was furthermore shown to be identical to the *fos* associated protein p39. To investigate the role of c-*jun* during mouse development we have generated mouse strains with ectopic expression of the c-*jun* gene (gain of function) and are attempting to inactivate the c-*jun* gene in ES cells (loss of function).

In order to study the consequences of overexpression of c-*jun in vivo* we placed the coding region of both the mouse and the human gene under the control of strong constitutively-expressed enhancer/promoter sequences, such as the human-b-actin promoter, the H2 promoter, the viral RSV and PCMV promoters (in collaboration with U. Rüther, EMBL, Heidelberg). The different c-*jun* constructs were introduced into fertilized eggs by DNA-microinjection and several transgenic lines were obtained. The exogenous *jun* gene was found to be expressed in several tissues of transgenic mice, however, no phenotype was yet observed. Introduction of some of these constructs into ES cells by electroporation led to the isolation of high expressing ES cell clones. High c-*jun* expression did neither alter the growth parameters nor did it change the undifferentiated ES cell morphology. These ES clones are presently being used for blastocyst injection and the generation of chimaeric mice.

In a complementary approach we are attempting to inactivate the c-*jun* gene by gene targeting through homologous recombination in ES cells in order to analyze the role of c-*jun* in mice lacking one member of the *jun* gene family.

III. Ectopic expression of transcription factor GHF-1 in transgenic mice

G. Veres, K. Schellander in collaboration with M. Karin (UCSD, San Diego).

The tissue-specific transcription factor, growth hormone factor-1 (GHF-1 or Pit-1) plays a critical role in pituitary-specific expression of growth hormone (GH) and prolactin. GHF-1 is a member of a family of DNA binding proteins containing a homeodomain and a second highly conserved sequence motif known as the POU domain. Expression of GHF-1 is restricted to two specific cell types in the anterior pituitary, the somatotrophs and lactotrophs. The GH 5' flanking region, which functions as a pituitary specific promoter element has two GHF-1 binding sites upstream of the TATA box both of which serve as functional upstream promoter elements in vivo.

We have generated transgenic mice containing the complete GHF-1 cDNA or a genomic GHF-1 fragment linked to heterologous promoter elements. The goal of these experiments is to investigate whether ectopically expressed GHF-1 is able to activate either endogenous GHF-1-responsive target genes or a coinjected reporter gene.

Three transgenic mouse lines expressing various levels of GHF-1 RNA in heart, skeletal muscle and tail from an RSV-GHF-1 transgene were established. The coinjected reporter gene, human GH-promoter linked to the CAT gene was also expressed in the same tissues suggesting that GHF-1 can activate the coinjected GH-promoter *in vivo*. Experiments are presently in progress to determine whether endogenous genes, e.g. growth hormone, prolactin are also being activated by the ectopically expressed transcription factor.

IV. Transgenic mouse models for human neurological disorders

A. Aguzzi and K. Gruber

Animal models for specific diseases are important for basic and applied research since an understanding of the molecular basis of disease and of novel therapeutical strategies can only be partly obtained in a clinical setting. Gene transfer methodologies in reconstituted organs and in transgenic mice are particularly useful for dissecting the individual steps of altered gene expression that underlie many diseases.

Deregulated expression of tyrosine kinase oncogenes is often found in central nervous system (CNS) tumours. We tested the pathogenetic relevance of these findings by deregulating the activity of oncogenes in neuroectodermal cells, since derangement of differentiation and tumourigenesis would suggest that the introduced alterations represent a cause, rather than a consequence of the observed pathologies. More recently we have applied a similar strategy to the study of neurodegenerative diseases.

1. *Expression of polyoma middle T in the nervous system of transgenic mice*

We have been characterizing transgenic mice (Nb mice) carrying the polyoma virus middle T (mT) antigen cDNA linked to the thymidine kinase promoter, which developed multiple neuroblastomas of the sympathetic nervous system between 2 and 3 months of age. Expression of the transgene (assayed by tyrosine kinase assay and *in situ* hybridization) was restricted to the neurons of the central and peripheral nervous tissue, probably due to a positional effect of the transgene integration. The phenotype arising in these mice has been characterized in detail and similarities to the human condition have been uncovered. Transplanted tumours and cell lines have been derived from the Nb mice and their properties are being characterized. We are currently determining the chromosomal localization of the transgene as well as cloning the site of integration.

2. *Expression of the neu oncogene in transgenic animals*

The *neu* (c-erbB-2) oncogene, a receptor-like tyrosine kinase with considerable homology to the EGF receptor, has been found to be frequently amplified and overexpressed in human mammary carcinomas. In addition, an activating mutation of *neu* is found in virtually all cases of peripheral neuroectodermal tumours (Schwannomas) induced perinatally in rats by chemical carcinogens, but not in concomitant tumours of different histological type. This observation points to a selective sensitivity of Schwann cells for the action of *neu*.

In order to study the molecular basis of this phenomenon, transgenic mice have been generated expressing the activated *neu* oncogene from a myelin basic protein (MBP) promoter, which should direct expression to oligodendrocytes and Schwann cells. In a parallel approach we are investigating the consequences of *neu* expression in a broad specturm of tissues. To this end *neu* has been fused to the TK and to the cytomegalovirus promoter and introduced into ES cells from which chimaeric mice will be produced. Presently we are analyzing *neu* expression in MBP-*neu* transgenic mice, in individual ES clones and are in the process of generating chimaeras.

3. *Expression of the human foamy virus in the brains of transgenic mice*

In a collaborative effort with Ivan Horak's group at the University of Würzburg we have analyzed the consequences of expression of the foamy virus genome in transgenic mice.

Human foamy virus (HFV) is a recently characterized retrovirus originally isolated from various human diseases. Although the prevalence of HFV was reported to be high in certain geographical areas, it has not been possible to identify HFV as the causative agent of any human disease. Like the HTLV and HIV viruses, the HFV genome has in addition to structural retroviral genes, a region containing three open reading frames, bel-1 to *bel*-3. Bel-1 transactivates HFV transcription. Transgenic mice carrying the *bel* region of HFV under transcriptional control of its own LTR develop a progressive degenerative disease of the CNS and of the striated muscle. Since expression of the transgene is closely correlated with the appearance of structural damage, the disease is likely to be caused directly by the HFV proteins. These findings might imply a previously unrecognized pathogenic potential of HFV in humans. In collaboration with Horak's group, we next want to conduct a detailed study on the development of the disease in the existing mice as well as to generate new transgenic mice using other promoters as well as defined regulatory genes.

V. Studies with ES cells

We have continued our investigations into the use of ES cells for generating "loss of function" mutations as well as for understanding the dominant effects on mouse development caused by the expression of polyoma middle T (mT) oncogene in ES cell chimaeras.

1. *Gene inactivation studies*

Inactivation vectors for *fos*, *jun* and IL-3 (all based on the positive/negative selection scheme) were used for gene targeting into several ES cell lines. Following Southern analysis of several hundred individual double resistant colonies, we were unable to identify a correct cell clone with the inactivated allele. Presently, we have altered our strategy and are attempting to obtain correct "knock outs" using promoterless *neo* constructs for *fos* and *jun* and an improved vector for the IL-3 gene.

2. *Molecular analysis of haemangioma development*

We have reported in the last two years that chimaeric embryos obtained by blastocyst injection of individual ES cell clones expressing mT were specifically arrested at mid-gestation when blood vessel formation was disrupted by multiple haemangiomas. These endothelial tumours were used to derive endothelioma cell lines (End) which, following injection into mice, rats, chicks and quails, induced the formation of host-derived haemangiomas. In an attempt to understand the molecular changes responsible for these phenomena, End cells were analyzed in an *in vitro* system (Fig.2). Within fibrin gels the End cells formed large haemangioma-like cystic structures indicating high fibrinolytic activity resulting from both increased production of urokinase-type plasminogen activator (u-PA) and decreased production of plasminogen activator inhibitors (PAI-1). Upon neutralization of excess proteolytic activity by protease inhibitors the aberrant morphogenetic behaviour of End cells could be corrected (Fig.2). These results suggest that a tightly controlled proteolytic activity is essential for the morphogenesis of normal vascular structures and that physiological protease inhibitors play an important regulatory role in angiogenesis. This work is done in collaboration with W. Risau's group in Munich and R. Montesano's group in Geneva.

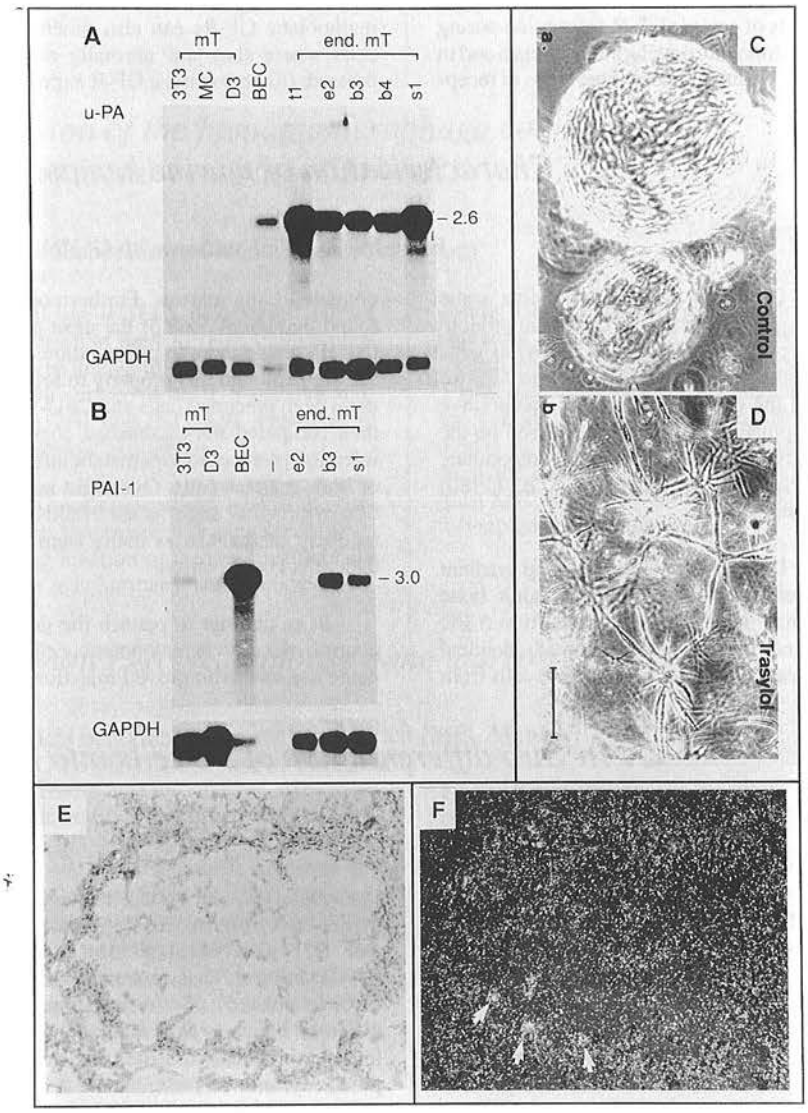

Figure 2. Effects of Polyoma middle T (mT) on endothelial cells.

u-PA (**A**) and PAI-1 (**B**) expression in various mT-expressing cell types. Endothelioma cell lines analyzed *in vitro* in fibrin gels in the absence (**C**) or presence (**D**) of protease inhibitors (Montesano et al., 1990). The aberrant morphogenetic behaviour (**C**) is corrected by neutralization of the increased proteolytic activity. **E,F**. *In situ* hybridization (using an mT-specific probe) of a haemangioma induced by subcutaneous injection of End cells into mice. (**E**) Haematoxylin and eosin stain; (**F**) dark field; only a small fraction of cells express mT (arrows).

VI. Hematopoietic stem cell development

This section describes a series of studies whose goals are to study the effects of expression of growth control genes, such as oncogenes and growth factor receptors on hematopoietic development in vitro and *in vivo*. Furthermore, attempts aimed at purifying hematopoietic stem (HS) cells and to differentiate ES cells into hematopoietic cells in vitro are being reported.

Hematopoietic growth factors and their receptors (GF-Rs) play a critical role in controlling proliferation and differentiation of hematopoietic cells. As one approach to study the function of various GF-Rs we are analyzing the biological effects of ectopic GF-R expression during hematopoietic development in vitro and in vivo. We have chosen three types of receptors:

(1) The multi-lineage receptor for the putative "stem cell factor" **(SCF-R)**, encoded by c-kit, present on early progenitor cells, the mast cell lineage and on erythroid cells.

(2) The lineage-restricted macrophage colony stimulating factor receptor **(CSF-1-R),** expressed only on the monocyte/macrophage lineage.

(3) The non-hematopoietic epidermal growth factor receptor **(EGF-R)**, which is normally not expressed on hematopoietic cells.

Specifically, we are interested in: (i) whether lineage-restricted or even non-hematopoietic GF-Rs can also function on cells where they are normally not expressed; (ii) how ectopic GF-R expression affects normal proliferation and differentiation; and (iii) whether it is possible to immortalize multipotent progenitor cells by supplying additional mitogenic signals via ectopically expressed GF-Rs.

At present, most protocols for manipulating HS cells involve retrovirus-mediated gene transfer into heterogeneous cell populations from adult bone marrow (BM) or fetal liver which consist mostly of multipotent and committed precursor cells but only of few HS cells (0.001%). An enrichment protocol for HS cells from total BM should therefore considerably improve the efficient gene transfer and expression of foreign genes during hematopoietic development.

1. *Characterization of murine hematopoietic stem cells*

F. Kiefer (in collaboration with G. Keller, Denver)

Our goal is to better define some physical properties of HS cells in order to improve the protocol for retroviral gene transfer. For the detection of these cells we used the extremely sensitive competitive repopulation assay which is based on the ability of HS cells to stably repopulate irradiated mice (Harrison, D.E. (1980) Blood, 55, 77).

Using a simple density step gradient we enriched HS cells from adult bone marrow 15-20 fold. HS cells from 5-FU treated bone marrow showed identical buoyant properties compared to cells from untreated bone marrow. Furthermore we found that about 50% of the most primitive HS cells adhere to tissue culture plastic. We exploited this property to separate them from precursor cells and CFU-S. We then compared the established coculture infection protocol to supernatant infection of bone marrow cells. Overnight incubation with virus supernatant results in a recovery of 5 times as many stem cells than after cocultivation.

In an attempt to perturb the growth control of early hematopoietic cells we made use of this improved injection procedure to introduce the polyoma middle (mT) oncogene into HS cells. By supernatant infection of density gradient enriched bone marrow with an mT-containing retrovirus we established a continuously growing cell line in vitro. In addition, mice were reconstituted with fractionated bone marrow which had been infected with the mT virus. Preliminary results from this experiment suggest that mT expression does not significantly perturb hematopoiesis early after reconstitution.

2. *In vitro differentiation of ES cells into hematopoietic lineages*

U. Burkert

The goal of this project is to define conditions that support the *in vitro* differentiation of ES cells into HS cells. ES cells can differentiate spontaneously in culture into multiple cell types, including various hematopoiteic lineages. Thus, these cells may offer the unique opportunity to analyze the earliest events of hematopoietic development *in vitro*.

Initial experiments were aimed at defining conditions for the *in vitro* differentitation of ES cells into hematopoietic lineages. Various ES cell lines were tested for their differentiation capacity. Following several days of culture in semi-solid media cells from two ES cell lines (CCE and D3) were able to form colonies (CFCs) *in vitro*. These colonies consisted of differentiated cells from a number of different lineages (fibroblast, epithelial, muscle) including those of the hematopoietic system. To better define the onset of hematopoiesis in this culture system, ES cells were differentiated for 22 days in the presence or absence of different growth factors. Every second day colonies were harvested and analyzed by histology and CFC assay. After 9 days, islands of red cells appeared in 10—30 % of the colonies, even in the absence of exogenous growth factors. However, histological analysis and CFC assay showed nucleated erythroid progenitors already at days 5 and 6. At later time points other hematopoietic lineages developed: monocytes (day 6), mast cells (day 13) and neutrophils (day 22). These results show that the ES cell system appears to be suitable for studying the function of growth factors and their receptors in hematopoietic stem cell differentiation.

3. *The role of c-kit in mouse development and the W phenotype*

W. Alexander

The c-kit gene encodes a protein typical of a transmembrane growth factor receptor. Although its function remains obscure, strong evidence suggests c-kit is allelic with the mouse W locus. Mutations at this locus produce pleiotropic effects manifest during embryogenesis, with animals carrying mutant alleles displaying defects in gametogenesis, pigmentation and haematopoiesis. Analysis of kit alleles in these mice revealed mutations which impair receptor function, strongly implying that c-kit encodes the receptor for an important developmental growth factor.

Our studies are aimed towards dissecting the role of c-kit in development and investigating the link between kit and W. Ectopic expression of the receptor in diverse cells via gene transfer should illuminate aspects of c-kit function. Moreover, the introduction of functional kit receptors into W mutants would prove and define the involvement of c-kit in this phenotype by the extent it cured or partially corrected the W defects. We have initially focussed on the haematopoietic system of W mutants, which is compromised by deficiencies in early stem and progenitor cells as well as in specific mature lineages, particularly erythroid and mast cells. Retroviral-mediated gene transfer can be utilized to deliver the normal murine c-kit gene to these affected cell populations.

We have constructed recombinant c-kit viruses (in collaboration with S. Lyman, Immunex, USA) and generated virus-producing fibroblast lines which express the exogenous c-kit protein and its associated tyrosine kinase activity. However, in virus-infected cells, including fibroblasts, DA-1 myeloid cells and primary mast cells, despite expression of the appropriate kit transcripts no receptor protein could be detected and no rescue of the growth defects in W mast cells has yet been observed. We next have to investigate why the viruses cannot transduce a functional receptor protein and are planning to construct new vectors for efficient kit expression in HS and ES cells.

4. *Ectopic expression of the human macrophage colony stimulating factor receptor (CSF-1-R)*

T. v. Rüden in collaboration with G. Mouchiroud (Lyon)

The receptor for human macrophage colony stimulating factor (CSF-1-R) was introduced into hematopoietic cell lines of myeloid and T-lymphoid origin, both of which normally do not express the CSF-1-R. Ectopic receptor expression on interleukin-3 (IL-3)-dependent mouse myeloid cells (FCD-P1), enables CSF-1-dependent short term and long-term proliferation. In contrast, no mitogenic signal was transduced in IL-2-dependent mouse cytotoxic T- cell line (CTLL), despite cell surface expression of the CSF-1-R. Furthermore, we were unable to detect the receptor on the cell surface of an IL-9-dependent T-helper cell line (ST2/K9) regardless of cytoplasmic expression.

These observations lead to the conclusion, that ectopically expressed CSF-1-R may function on certain myeloid cells where it is normally not expressed, suggesting the presence of signal transduction pathways, which can be utilized by the foreign receptor. In contrast, it appears that T-lymphoid cells lack such a signalling mechanism, indicating that quite different modes of transducing mitogenic signals from the cell membrane to the nucleus must have developed during myeloid and T-lymphoid differentiation.

5. *Expression of human EGF-R in mouse bone marrow cells*

T. v. Rüden and S. Kandels in collaboration with A. Ullrich (MPI, Munich)

Initial experiments have shown that the human EGF-R is capable of transducing EGF-dependent mitogenic signals on primary IL-3-dependent mouse bone marrow cells (BMC) and FDC-P1 cells following retroviral gene transfer (I.M.P.-Workshop 1988). Last year we have reported the extension of such studies to other hematopoietic lineages. While the EGF-R can function on primary mast cells and various IL-3-dependent cell lines, no mitogenic signal was transduced in either CSF-1-dependent macrophages or in IL-2-dependent T-lymphoid cells. Here, we report the biological consequences of expressing the normal human and mutated EGF-Rs on mouse BMC in reconstituted mice.

Transplantation of BMC expressing the human EGF-R did not lead to an alteration of hematopoietic development. However, we were unable to detect any surface expression of the EGF-R in long-term reconstituted mice, despite the presence of intact proviral DNA. Although we have no direct proof, evidence obtained from in vitro experiments suggests a selection against cells expressing the foreign receptor molecule.

In contrast, mice reconstituted with BMC expressing the oncogenic viral homolog, v-erbB, developed a systemic mast cell disease 8—12 weeks after transplantation. The disease is characterized by a dramatic infiltration of connective tissue type mast cells in bone marrow, spleen and several non-hematopoietic organs, such as liver, kidney, heart and intestine; thymus, brain and spine were not affected. The observed pathology can best be described as Malignant Mastocytosis. Transfer of BMC or spleen cells from such mice into secondary recipients leads to the development of acute leukemias three weeks after transplantation. Initial characterization of the leukemic cells exhibited the presence of myeloid and lymphoid markers and in liquid culture such cells have the capacity to differentiate along the monocytic lineage.

6. *Characterization of mouse erythroblast cell lines dependent on erythropoietin for proliferation.*

G. Keller and M. Kennedy in collaboration with H. Beug

In 1989 we reported the establishment of v-src oncogene expressing mouse cell lines that could be grown in the presence of erythropoietin (EPO). Several sublines were generated which exhibited a proliferation response to human recombinant EPO, allowing the development of a sensitive EPO bioassay. Apart from a weak response to IL-3, no other known hematopoietic growth factors significantly supported the growth of these cells. The cells have been characterized belonging to the erythroid lineage, since they express a mammalian homolog of the erythroid-specific avian histone H5, they can be induced to express hemoglobin in > 70% of the cells by chemicals (butyric acid) and are negative for a whole series of myeloid or lymphoid cell surface antigens. They clearly differ from Friend erythroleukemia cells since drugs inducing differentiation in the latter (e.g. DMSO) are completely inactive in v-src erythroblasts.

From variant cell lines that were able to grow independently of exogeneous growth factors at high density (see report 1989), a subline was isolated that produced significant amounts of a growth factor active both on factor-dependent src-erythroblasts and on normal bone marrow. Trials to characterize this activity have so far yielded somewhat ambiguous results: while we were unable even by very sensitive techniques (PCR) to detect mRNA for known factors (GM-CSF, IL-3) in these cells, the growth factor activity secreted by these cells could partially be neutralized by IL-3 antibodies. It is thus possible that the cells produce IL-3 plus an unknown factor. We are currently testing whether or not the latter might represent the recently identified ligand for the c-kit proto-oncogene, since all src-transformed erythroblast lines examined expressed kit associated tyrosine kinase activity at high levels.

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II. The Group Leaders

The role of transcriptional control in the pheromone response in yeast

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Hans WINKLER	Postdoc
Michael PRIMIG	PhD-student
Claudia PETRITSCH	diploma student

Conjugation as a developmental choice in yeast is normally limited to haploid cells. It requires two transcriptionally regulated processes that (A) establish intercellular communication and (B) induce conjugation specific programs when the appropriate signal has been received from the potential mating partner. The system provides an excellent model to examine the problem of how combinations of a limited set of transcriptional regulators can establish a cell autonomous program that allows a cell to respond to certain environmental cues.

The function of STE12 as a transcription factor

G. Ammerer, M. Primig, C. Petritsch, B. Errede*)

The STE12 product is essential at several steps in the mating process of yeast. It is necessary for high level expression of pheromone and receptor genes as well as the induction of conjugation specific functions. We have shown previously that the gene encodes a DNA-binding protein which recognizes a DNA sequence commonly referred to as pheromone response element (PRE). In multiple copies this element is sufficient for the creation of a pheromone inducible promoter. Therefore, it is likely that the STE12 protein constitutes one of the nuclear targets of the pheromone dependent signal transduction pathway. This proposal is further supported by the observation that so far all STE12 dependent promoters also require the integrity of the signal transduction pathway.

In vitro binding studies indicate that the complete STE12 protein as synthesized in a cell free extract binds only weakly to its recognition sequence, a single PRE. However, under two conditions we observe increased binding of the protein: (A) A truncated version of the protein lacking up to two thirds of its C-terminal amino acids can bind with high affinity. (B) The full length protein can bind in cooperation with MCM1, a protein involved in the activation and repression of cell type specific genes. The result suggests that C-terminal domains of STE12 exert a negative affect on DNA binding perhaps by obscuring the DNA binding domain or by altering its conformation. This negative function can be neutralized at least in one case by the interaction of STE12 with another DNA binding protein. Additionally, it is possible that STE12 binding is modulated by the introduction of posttranslational modifications. Indeed, we have been able to show that STE12 is a phospho-protein and that phoshorylation is required for DNA binding. Currently we are investigating the effect of pheromone induction on the phosphorylation status of the protein.

High level expression of theSTE12 gene can be detrimental for cell growth. One specific consequence of unphysiologically high levels of the protein is the transcriptional induction of pheromone-inducible genes independent of an external signal. In parallel STE12 overexpression also suppresses the transcriptional defects of mutations in upstream components of the signal transduction pathway. However, such mutants remain sterile under these conditions indicating that some mating functions are regulated by post transcriptional mechanisms or by transcriptional events independent of STE12. One important consequence of the pheromone response concerns the arrest of the cells in the G1 phase of their cell cycle. The question whether STE12 overexpression will lead to similar events in respect to cell cycle arrest remains to be determined.

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Identification of a MCM1 domain sufficient for its cooperation with cell-type specific activators and repressors

M. Primig, G. Ammerer

MCM1 encodes a DNA binding protein essential for the survival of the yeast cell. The protein is also involved in the activation and repression of genes that confer mating specificity to a normal haploid cell. In the context of these cell type specific promoters MCM1 binds in cooperation with other DNA binding proteins, the activators alpha1 and STE12 and the repressor alpha2. MCM1 is closely related to the mammalian transcription factor SRF (serum response factor), a fact that is also reflected by the similarity of their target sequences. Comparing the two proteins one can find a bipartite 90 amino acid domain with almost 70% identity between SRF and MCM1. This domain provides the oligomerization and DNA binding functions in both proteins (the sequence is unrelated to the DNA binding and dimerization motifs of other families of DNA binding factors). It has been proposed that SRF has to interact with additional DNA binding proteins to exert its biological function, e.g. in relation to growth factor stimulated signal transduction. The situation may be analogous to the interaction between STE12 and MCM1.

We constructed a truncated version of MCM1 retaining only the part encoding the domain with similarity to SRF. Expression of just this 98 amino acid domain is able to rescue a strain from the lethal effects of a mcm1 deletion. Surprisingly, such a strain also exhibits normal mating specificity. In vitro DNA binding assays confirm that the truncated protein is still able to interact with alpha1, alpha2 and STE12. We replaced the sequences proposed as important for dimerization of MCM1 with the equivalent sequences of SRF. The hybrid gene is still able to provide for the essential functions of MCM1. However, strains containg the MCM1/SRF hybrid are completely sterile in both haploid cell types. This result is in agreement with DNA binding data which indicate that the hybrid protein binds to the MCM1 target sequence but is barely or not at all able to recruite alpha1, alpha2 and STE12 into the DNA/protein complex. These data suggest that the interactions between MCM1 and these factors depend on specific contacts within the dimerization motif.

Both the activator STE12 and the repressor alpha2 have to cooperate with MCM1 on a-specific promoter elements in order to exert their biological function. Using in vitro binding experiments we found these interactions to be mutually exclusive. It suggests that the repressor might also function by blocking access of the dimerization domain of MCM1 to other transactivators. This might serve as a convenient model for the negative regulation found at serum responsive elements in higher eukaryotes.

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Regulation of protein serine/threonine kinases during early G1

Lisa M. BALLOU Group Leader

Introduction

Addition of growth factors or expression of oncogenes in quiescent fibroblasts leads to a large increase in phosphorylation of proteins on serine (S) and threonine (T) residues. This is mediated by the activation of S/T-specific kinases and/or inhibition of S/T-specific phosphatases. Several mitogen-activated kinases have been identified, including S6 kinase and MAP2 kinase. S6 kinase is thought to phosphorylate the S6 protein of 40S ribosomal subunits in the intact cell. This event is associated with an increased rate of translation that is required for progression through S phase. The major S6 kinase in fibroblasts is an enyzme of MW=70,000. It is activated *in vivo* by phosphorylation of S/T residues by an unknown S6 kinase kinase and can be inactivated *in vitro* by treatment with protein phosphatases 1 or 2A. Thus, it appears that S6 kinase participates in a phosphorylation cascade initiated by growth factor receptors, with at least one S/T kinase linking these two enzymes. MAP2 kinase is another mitogen-activated kinase in fibroblasts. This enyzme (MW=42,000) is unique in that it is thought to be activated by a combination of T and tyrosine (Y) phosphorylation. Although the physiological target of MAP2 kinase is not known, it has been suggested that this enyzme is also involved in the cascade that leads to S6 phosphorylation. I am interested in identifying the S6 kinase kinase, with the ultimate aim of tracing all the steps linking receptor tyrosine kinases and S6 kinase.

Identification of S6 kinase kinase

A strategy using both *in vitro* and *in vivo* approaches must be used to search for S6 kinase kinase. The *in vitro* approach is based on an assay that will detect kinases that phosphorylate and reactivate S6 kinase. Briefly, the active S6 kinase will be purified, then dephosphorylated and inactivated with phosphatase 2A. Then the protein will be incubated together with ^{32}P-ATP and the kinase of interest and we will assay for reappearance of S6 kinase activity and phosphorylation of the MW=70,000 protein. Two sources of test kinases will be used: (1) purified enzymes that are known to be activated early in G_1 and (2) fractionated extracts from resting and stimulated cells. I assume that the kinase kinase will only be active in fractions from stimulated cells. An important aspect of this assay is to ensure that the S6 kinase remains stable during all of the incubations. Otherwise, false positives are frequently obtained. Conditions have now been established to prevent this artefact.

In conjunction with the above assay, in vivo responses of the putative kinase kinase will be examined. For example, any treatment resulting in S6 kinase activation must also cause increased activity of the kinase kinase. In addition, any inhibitor that blocks the activity of kinase kinase will prevent activation of S6 kinase (see following section). A positive result from both of these approaches will allow us to construct a plausible in vivo kinase cascade.

The above strategy was recently used to test whether MAP2 kinase could be S6 kinase kinase. This enzyme seemed to be a particularly good candidate because it was thought to be activated by Y phosphorylation and, on the basis of *in vitro* results, Gregory et al claimed that it could activate S6 kinase. We found that EGF activates MAP2 kinase faster than S6 kinase in fibroblasts (maximal at 2.5 min *vs.*10 min), as would be expected if the former enzyme activates the latter. However, MAP2 kinase did not reactivate S6 kinase *in vitro*, nor did it phosphorylate the enzyme. In addition, we found that insulin treatment of fibroblasts does not activate MAP2 kinase but does lead to increased S6 kinase activity. Thus, MAP2 kinase cannot be the activator of S6 kinase.

Inhibitors of S6 kinase activation

An increased level of cAMP early in G_1 antagonizes the growth of fibroblasts. Resting cells treated with 8BrcAMP or prostaglandin E_1 show a slight increase in S6 phosphorylation, due to activation of cAMP-dependent protein kinase, but no increase in protein or DNA synthesis. Interestingly, pretreatment of cells with cAMP phosphodiesterase inhibitors such as theophylline or SQ20006 completely blocks the EGF-induced phosphorylation of S6. SQ20006 does this by preventing activation of S6 kinase. The site of action of SQ20006 is unknown. Since the drug has no effect on activation of MAP2 kinase, it problably does not act as a general kinase inhibitor. This and other related compounds may be useful tools in identifying participants in the pathway leading to S6 kinase activation.

Novel mitogen-activated kinases

Although the MW=70,000 enzyme is the major S6 kinase identified in fibroblasts, *in vitro* this kinase modifies only 4 of the 5 phosphorylation sites in S6. This suggests that full phosphorylation of the protein *in vivo* may require the cooperative action of more than one kinase. Indeed, two additional mitogen-activated S6 kinases have now been detected in fibroblasts. One is immunologically related to S6 kinase I and/or II from *Xenopus* oocytes (MW= 90,000 and 92,000) and is maximally active very early after EGF addition (2.5 min, as opposed to 10 min for the MW=70,000 enzyme). The second kinase activity is only detected at late times (1 hour) and can be separated from the others by chromatography on Mono S. Preliminary characterization of this S6 kinase suggests that it may be related to the MW=70,000 enzyme. The interrelationship between these enyzmes and their possible contribution to S6 phosphorylation *in vivo* will be studied.

Regulation of S6 kinase by phosphatase 2A

The level of S6 kinase activity *in vivo* reflects competition between S6 kinase kinase and S6 kinase phosphatase. The major kinase phosphatase in fibroblasts appears to be a type 2A enzyme, which consists of a catalytic subunit bound to two or three proteins that are presumed to be regulatory subunits. Several observations suggest that the activity of this enzyme might be regulated differentially by mitogens. For instance, S6 kinase activity in cells treated with EGF is switched off very rapidly as compared with cells treated with insulin. This could be due to stimulation of phosphatase 2A. Confirmation of this with an *in vitro* assay would be the first demonstration that phosphatase 2A activity is controlled.

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Mammalian Developmental Genetics

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This group is interested in studying genes that regulate mammalian development. Our approach is to use reverse genetic strategies to clone the genes responsible for naturally occurring mouse mutations. The t complex on chromosome 17 which exists in two structural forms in wild type mouse populations (wild type and t haplotype) is now one of the best understood and characterized genetic systems in the mouse. Furthermore it is a region of the genome in which a large number of developmental mutants have been identified that are potentially accessible by molecular genetic techniques. We are directing our efforts at present to the analysis of a 4 megabasepair region in the proximal part of the t complex that contains 5 developmental mutants (**see Figure 1**). Progress so far has resulted in the identification of candidate genes for the *Tme* and the *Tcd*-3 mutations, and in the isolation of a novel gene from the *Tcr* region. Future goals for the next year will be to use both the mutant and the wild type mouse to tell us how these genes function during mammalian development.

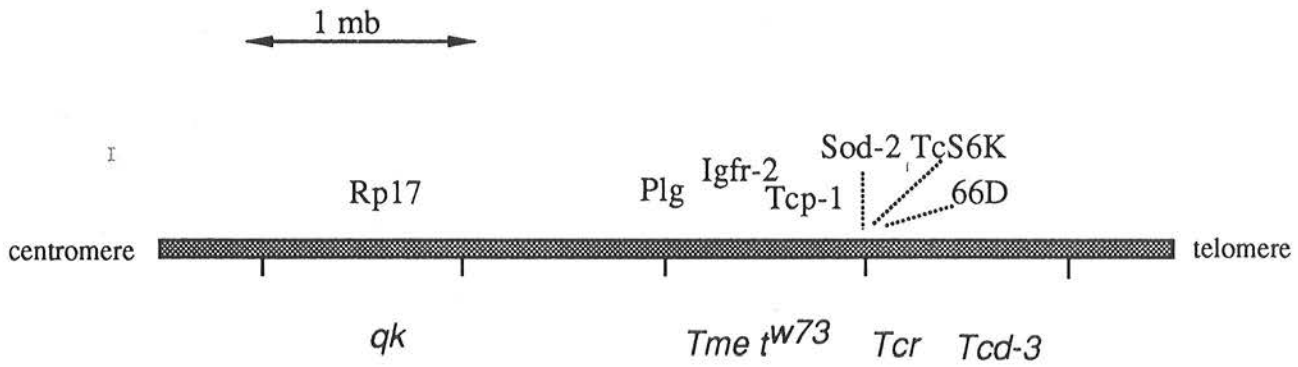

Figure 1: Physical map of a 4 mb region from the mouse t complex showing the position of DNA markers (above the line) and of five developmental mutants (below the line).

Identification of an imprinted gene at the Maternal-effect locus.

D. P. Barlow, R. Stöger, K. Saito, B. G. Herrmann and N. Schweifer.

Tme (**T**-associated **m**aternal **e**ffect) is the only known maternal-effect mutation in the mouse. The defect is known to be encoded within the embryonic genome and not caused by uterine or oocyte-ctyoplasmic defects. Embryos that inherit a deletion of the *Tme* locus from their mother die at day 15 of development in contrast to viable and fertile embryos that inherit the same deletion from their father. Although the biological role of genomic imprinting is unknown, there are six known imprinted chromosomal regions in the mouse and it is possible that imprinting may contribute substantially to the regulation of gene expression during development. No endogenously imprinted genes have been cloned and thus no information has yet been obtained about the molecular basis of this phenomenon although analogies with X-chromosome inactivation in females have been made. Our approach to isolate *Tme* involved a combination of mapping and cloning techniques using two chromosome 17 deletion mutants that lack the *Tme* gene, T^{hp} and t^{wlub2}. This year we have shown that three genes, insulin-like growth factor-2 receptor, superoxide dismutase-2 and plasminogen, map within or close to the t^{wlub2} deletion. A physical map of these genes using pulsed-field gel electrophoresis, in combination with a genetic analysis of the deletion chromosomes, showed that *Tme* must lie within an 800—1100 kb region. RNA blot analysis of maternal-effect mutant embryos has furthermore identified insulin-like growth factor-type 2 (Igf2) receptor as expressed only from the maternal chromosome while flanking genes are expressed from both chromosomes. Thus the Igf2 receptor is imprinted and closely linked or identical to *Tme*. Our goals for the further study of the *Tme* mutation are to use the Igf2 receptor as a model gene to examine the molecular basis of genomic imprinting. Questions will be asked about the onset of differential gene expression from the maternal and paternal chromosomes during gametogenesis and embryogenesis, the upstream regulatory sequences that may form the imprinting signal, the size of the imprinted region, and in the future, rescue or inactivation experiments will be performed to test directly whether the Igf2 receptor is the *Tme* gene.

A novel S6 kinase gene maps to the mouse t complex.

R. Stöger, A. Kispert, B.G. Herrmann and D. P. Barlow

Cosmid clones isolated from a walk at the distal end of the t^{wlub2} deletion were analysed for the presence of new genes by hybridization to RNA blots prepared from 20 different adult and embryonic tissues. In this way a novel S6 kinase gene named provisionally TcS6K, has been identified. S6 kinase phosphorylates the S6 protein of the large ribosomal subunit and this step may play a role in the increased translational activity cells show after transformation. Two other mouse S6 kinases are known and these show high homology but are clearly different to TcS6K.This novel kinase is expressed in many tissues including the early embryo and placenta, adult muscle and testes.

Recombination between wild-type and t-haplotype chromosomes at the Plasminogen locus.

N. Schweifer and D.P. Barlow

Plasminogen is the precursor of plasmin, a serine protease involved in fibrinolysis. The gene has been mapped to the proximal part of mouse chromosome 17 between *Rp17* and *66D* (Figure 1 and Degan et al., Genomics 8, 49-61, 1990). These two loci delineate the position of the t^{w73} mutation which is lethal during implantation, a process known to require proteases. We have analyzed the *Plasminogen* locus in the wild type and t haplotype chromosomal variants and in the partial t haplotypes, t^{wlub2} and Tt^{orl}, that appear to be reciprocal chromosomes generated by recombination between + and t chromosomes. Recombination between + and t chromosomes is normally suppressed throughout the region known as the t complex due to the presence of multiple inversions but rare recombinants can arise. We show that the *plasminogen* locus is present in multiple copies on the t chromosome in orientations that would allow homologous recombination across paired inverted regions and that the plasminogen locus is disrupted in the t^{wlub2} chromosome. The t^{w73} gene is deleted in t^{wlub2} but not in Tt^{orl}. Expression analysis by RNA blots suggests however that only one locus is expressed in t haplotypes and furthermore that expression is not altered in heterozygous t^{w73}/+ mice. Further work will continue the search for t^{w73} by extending a cosmid walk from the plasminogen locus into the t^{wlub2} deletion.

A candidate gene for a mouse t-specific distorter locus.

B. Alexander and D. P. Barlow

Male mice heterozygous for the t and wild type forms of chromosome 17 produce gametes that carry either chromosomal variant. Despite this, up to 99% of the progeny are derived from t-carrying gametes. This drastic alteration in transmission ratio is regulated by t-specific alleles of three distorter loci that act additively on a single responder locus. We have identified a gene expressed only in testicular germ cells during the onset of meiosis. The gene named Tcte-2 shows tight genetic and physical linkage to the Tcd-3 (t complex distorter-3) locus and mRNA analysis shows that expression is reduced in testes of t/+ heterozygotes and absent from testes of t/t homozygotes. Based on these three criteria (linkage, expression pattern, altered expression in mutant animals) we propose that Tcte-2 is a candidate gene for the Tcd-3 mutation. Immediate goals will involve the isolation of full length clones for Tcte-2, and the characterization of the wild type and t haplotype regulatory regions in order to understand the absence of expression in the mutant. Examination of the testes expression pattern in testes from wild types and heterozygote mice will also be continued in our attempt to correlate Tcte-2 with changes in gamete transmission ratios.

Genetic and physical mapping of mouse chromosome 17

D. P. Barlow, in collaboration with J. Nadeau and H. Lehrach.

An accurate chromosomal map containing a high density of DNA markers is a prerequisite of all reverse genetic approaches to gene cloning. This is especially important in the mammalian genome where genome size (3000 mb), intergenic distances (60—100 kb) and the presence of repetitive sequences make it necessary but laborious to cross large genomic distances. To this end we are also involved in a continual process of updating the genetic and physical map of chromosome 17. Part of this work has involved the establishment of a committee in collaboration with other workers interested in this chromosome, and one role of this committee will be to provide an annual update of markers mapped to this chromosome.

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AP1 Antagonists as Tools to Study Cell Growth and Differentiation

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Introduction

The products of the oncogenes c-*fos* and c-*jun* (Fos and Jun) have been found to be components of the transcription factor AP1 (for example, see Rauscher et al., 1988) which is believed to mediate phorbol ester responsive stimulation of transcription through its target sequence, the TRE (tetradecanoyl phorbol acetate responsive element). The TRE appears to be a specific target for protein kinase C-dependent regulation of transcription. AP1 consists of a number of Fos and Jun homologues, which are differentially regulated (for a review, see Curran & Franza, 1988). It is becoming clear that there are also considerable differences in the functions of different members of each homologous group (Chiu et al. 1989, Lucibello et al., 1990). AP1 thus represents a dynamic and complex system of transcriptional regulators. It has been proposed that modulation of the activity of AP1, through changes in the activity or concentration of its components, may be central in coupling signal transduction to longer term changes in gene expression.

We are attempting to study the biological functions of AP1 by interfering with its activity *in vivo*. To this end, we are designing dominant interfering proteins, which when expressed in vivo, reduce the activity of all or of selected AP1 components. Expression of such proteins will be placed under control of regulatable promoters.

Design of proteins that interfere with AP1 function.

Dominant interfering proteins are altered gene products which retain the wild-type protein's affinities for limiting target molecules (substrates, other proteins and DNA, for example), but are inactive or weakly active in the effector function of the wild-type protein. We are designing dominant interfering proteins based on the sequences of the Fos and Jun proteins. AP1 appears to contain protein dimers; either heterodimers of Fos homologues with Jun homologues, or of homodimers of Jun homologues (Nakabeppu et al., 1988). The DNA-binding domain of a dimer appears to be composite, with one half provided by each monomer. Association between monomers appears to be mediated entirely by the "leucine zipper" domain (see O'Neil et al., 1990). Fos and Jun homologues, therefore, have two separable functional domains which can be exploited to make dominant interfering proteins.

We are designing dominant interfering derivatives of Fos and Jun which retain both the DNA-binding domain and the leucine zipper, but we have eliminated their transcriptional activation domains. Such proteins should be able to associate with TRE's, competing with functional endogenous AP1. Derivatives of this sort will only work if the single transcriptional activation domain of the normal "partner" is inactivated or otherwise insufficient for the complex to function as a transcriptional activator.

Alternatively, expression of a protein that contains only a leucine zipper and no DNA-binding domain (or an inactivated one) should lead to formation of a complex between the leucine zipper and its normal cellular partner, preventing that partner from binding to DNA. We have also been developing constructions of this sort, but have not yet found them to function in NIH3T3 cells. Recently, Jun mutants lacking their DNA-binding function have been described. They interfere with Fos-activated transcription in F9 embryonic carcinoma cells, which have very low endogenous AP1 activity (Smeal et al., 1989; Ransone et al., 1990). We suspect that the instability of the natural Jun protein will be reflected in its mutants, consequently it may be difficult to express sufficient mutant Jun protein in cells that contain higher endogenous levels.

Fusion Proteins Containing both the Leucine Zipper and the DNA binding Domain.

We have produced a dominant interfering Jun derivative which has proved promising *in vivo*. We have fused the coding sequence of a small, stable, basic, monomeric protein with the DNA-binding domain and leucine zipper of the Jun protein, creating the DBZj protein. This protein binds the TRE specifically *in vitro* and forms DNA binding complexes with Fos. We have also exchanged the DNA-binding domain and leucine zipper of Jun in this protein for the homologous region of Fos, to create DBZf. DBZf forms complexes with Jun and with DBZj which bind DNA, but like Fos itself, it does not have specific affinity for DNA. We have not yet tested DBZf in vivo.

So far, we have investigated the DBZj chimaeric gene for its effects *in vivo*. It is expressed when transfected into NIH3T3 cells, and inhibits expression from a TRE-activated reporter gene. DBZj also antagonizes the activation of the TRE-reporter gene by cotransfected *fos*. In both cases the effects are approximately 50%. For comparison, in both cases, we used a mutated construction, Dx, in which there is a frame shift between the carrier protein and the Jun portion of the gene.

To study the effect of constitutively expressing DBZj, DBZf and Dx we constructed retroviral expression vectors that also carried the G418 resistance marker. We have readily derived a Dx-expressing virus-producing line, but to date we have been unable to produce a virus producing line from the potential dominant interfering constructs, hinting that expression of DBZj may interfere with the establishment of cell-lines. Moreover, in calcium phosphate transfected NIH3T3 cells, the Dx vector was six times more effective than the DBZj in forming colonies. Linearizing the DBZj vector doubled its efficiency, whereas linearizing the DBZj vector and cleaving the 5' LTR (the promoter) from the end of the DBZj gene (which is expected to inactivate the gene) increased the efficiency of colony formation a further six-fold. These data are consistent with a hypothesis that expression of the DBZj protein is blocking essential genes that are required for colony formation, perhaps at the level of cell proliferation.

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A single site mutation in a truncated Fos protein allows it to interact with the TRE in vitro

Martin Nicklin and Georg Casari.

We show that a single amino-acid change in the leucine zipper of Fos is sufficient to allow a truncated Fos protein to homodimerize and thus form a complex with DNA, even in the absence of Jun. This Fos-derived homodimer recognizes the TRE specifically *in vitro*. We conclude that the structural requirements for specific DNA binding are present in the Fos protein itself, with the exception of its lack of self-affinity.

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New Cell Cycle Regulated Genes In Saccharomyces Cerevisiae

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During the past few years the investigation of the cell cycle has led to a better understanding of how a cell regulates its progression through the cell cycle. Initial work on frogs and marine invertebrates indicated that the maturation promoting factor (MPF) represents the main component whose oscillations drive the mitotic cell cycles of early embryos. Only after studies on the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* uncovered the linkage between the cdc2/CDC28 gene products, $p34^{cdc2}$, and MPF, a general concept of regulation of the cell cycle evolved. $p34^{cdc2}$ which has been identified as a serine/threonine kinase plays a central role in the regulation of the cell cycle. It forms a complex with a second component known as cyclin to generate MPF. A dephosphorylation step leads finally to the active form of the kinase which then forces the cell to cross the boundary between the cell cycle stages G2 and mitosis. This step is accompanied by the decay of MPF. Studies of the cell cycle in S. *cerevisiae* have revealed a second crucial regulatory point, besides the G2/M transition point, which resides at the boundary between G1 and S phase. A cell which traverses through this point, called START, is committed irreversibly to complete the next cell cycle. The current model suggests that at this point the $p34^{cdc2}$ kinase forms complexes with a set of three proteins, which are collectively known as the G1 cyclins on the basis of limited sequence similarity to the previously characterized cyclin family. These G1 specific complexes are thought to form an active kinase of different substrate specificity to the G2/M kinase. The activity of this kinase which has been found, so far, only in S. cerevisiae finally enables the cell to pass through START.

Although the information outlined above led to a better understanding of the events which control crucial regu- latory points in the cell cycle, the question still remains open as to how the timing of these events is regulated. The data elucidated so far support a concept of post-translational mechanisms which act due to modifications of proteins involved in the control events. One intriguing possibility is that transcriptional controls play a key role in the correct ordering of the discontinuous processes of the cell cycle. In this conception certain genes would be expressed only at specific points in the cell cycle and their gene products would interact with the already known posttranslational events. Most of the already identified cell cycle regulated genes in *S. cerevisiae* are involved in the DNA replication machinery, DNA repair, and in the mating type switching. The G1 and G2 cyclins are the only examples of genes so far whose transcription is cell cycle dependently regulated, as we were able to show, and which are also involved in the control or timing of the cell cycle. In order to achieve a better insight into the complex control mechanisms of the cell cycle we decided to identify more cell cycle regulated genes and search for those which are involved in the controls of the cell cycle.

A general approach to the isolation of new cell cycle regulated genes

The use of standard genetic approaches such as the creation of temperature sensitive cell division cycle mutants has not led to the identification of cell cycle regulated genes which are an integral part of the control mechanisms of the cell cycle. One possible explanation for this failure may be that many genes are refractory to conventional mutagenesis. Redundancy of genes can also impede their recognition by standard genetic analysis; the cyclin genes are examples for this. Since we sought to find novel genes of this kind we decided to develop a new approach which would detect most, if not all, cell cycle regulated genes. Available techniques, such as differential hybridization or dot blot analysis, did not promise to be sensitive enough for our purpose. The approach we finally decided to use relies on the existence of an ordered lambda phage library provided by M. Olson, which encompasses 80 to 90 % of the haploid yeast genome as minimal overlaps in 855 phages. The internal yeast DNA segments have been mapped according to their EcoRI/HindIII restriction pattern. We use the individual DNA restriction fragments to probe Northern blots which were derived from polyadenylated RNAs separated by formaldehyde/agarose gel. These RNAs were isolated from yeast cells out of six different cell cycle stages, thus enabling us to differentiate easily between unregulated and regulated transcripts.

Using this primary screen we have been able to examine 55% of the library so far using more than 4000 Northern hybridizations, which detected around 2000 different transcripts. The analysis of the data derived from 300 phages, which encompasses 60 % of the total data of our primary screen, has identified approximately 160 candidate transcripts which show cell cycle dependent regulation.

In order to confirm the data accomplished using the primary screen it is important to establish that the transcripts exhibit temporal periodicity in exponentially growing synchronous cultures. Our secondary screen analyses the behavior of the previously identified transcripts in cells which are first arrested at a specific point in the cell cycle and then released from the cell cycle block. Samples are then removed from these cultures at successive time points and the RNA analysed by Northern blots using the same DNA probes as for the primary screen. This screen identified 20 transcripts out of 40 as being clearly cell cycle regulated. The rest were false positives. We are currently analysing a further 120 candidate transcripts in the secondary screen. By analogy to our previous data we would expect at least 60 of these 120 to be genuinely cell cycle regulated. Assuming that such transcripts are randomly distributed in the genome we would predict that approximately 200 to 250 loci exist which direct the synthesis of cell cycle regulated transcripts.

Identification and characterization of transcripts

In order to identify genes, whose transcription exhibits a characteristic cell cycle dependent fluctuation, we subclone the DNA segments and sequence them partially. The comparison of the derived peptide sequences with databases tells us whether this DNA represents a gene or part of a gene which has already been identified. It helped us to identify known genes like HTB1 (histone H2B), TOP2 (topoisomerase 2), RNR1 (large subunit of ribonucleotide reductase), and CLN1 (G1 cyclin 1). We have also identified several unknown genes. While most of them exhibit expression at the late G1/S boundary, one gene showed strong expression at the stage between mitosis and early G1, another one in mitosis. This is the first time that cell cycle regulated transcripts have been assigned to these two cell cycle points. Since there are no good cytological markers in the area between mitosis and G1, the onset of transcription of the former of the two genes will serve as an important landmark for entry into G1.

A further step in the process of characterization involves the test of essentiality. Only one gene tested so far by disruption experiments turned out to be essential for life of the cell. This gene was identified as a low abundance transcript which also seems to fluctuate with the expression of the mitosis specific transcript. The remaining genes do not seem to be essential in our tests, because they are either functionally redundant or do not exhibit functions, which can be detected under laboratory conditions as being important for the cell life cycle.

MST1 and MST2: Two highly related genes encoding putative membrane spanning proteins

MST1 (**m**itosis **s**pecific **t**ranscript) represents the gene which is highly expressed in mitosis. We chose this gene for more detailed studies because of its unique transcriptional characteristic. Gene replacement showed that it was not essential for viability. Subsequent tests using Southern blots under non-stringent conditions indicated the existence of a structural analogue. This was isolated by dot blot analysis of the ordered phage library supplied by M. Olson. The DNA sequences revealed that these were two previously uncharacterized genes encoding proteins with seven hydrophobic domains each with a length sufficient to span a membrane. Since the last five hydrophobic domains show similarities to the last five hydrophobic domains of the light-driven proton pump bacteriorhodopsin, it is possible that both genes encode membrane spanning proteins performing ion channel functions. Initial results of gene disruption experiments which simultaneously inactivated both genes indicate that this is also not a lethal event. Despite this these genes seem to be conserved in evolution, since homologous sequences have been identified in the DNA of *Klyveromyces lactis* and *S. pombe* by Southern blots.

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Biological Chemistry

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Transferrin-polycation DNA complexes: the effect of polycations on structure of the complex and DNA delivery to cells.

Ernst Wagner with Matt Cotten and Max L. Birnstiel

In order to adapt the natural iron transport system for DNA delivery to cells, we have synthesized conjugates of the iron transporter transferrin with various polycations (Wagner et al., 1990). Transferrinfection, the efficient cellular uptake and expression of DNA complexed with transferrin-polycation conjugates, has been shown to be based on transferrin-dependent receptor-mediated endocytosis (Cotten et al., 1990; Zenke et al., 1990) with the polycation polylysine (pL) or protamine acting as the DNA-binding moiety. The optimized ratio of transferrin-polylysine (TfpL) conjugate to DNA was found to be near or equal to the ratio that results in electroneutrality of the complex. Unconjugated polycations of the same type had no significant transfection activity.

Various new TfpL conjugates have been synthesized with improved procedures that resulted in higher yields and transfection efficiency . In DNA delivery experiments we found that polylysine-rich conjugates are more efficient in delivery of pRSVL plasmids into K-562 cells. Electron microscopy studies with these samples revealed a profound condensation of the plasmid DNA to toroid structures (doughnuts) with a size of about 80-100 nm in diameter (see **Fig.1**). It appears then, that one property of the TfpL/DNA complex is a condensed structure that may facilitate the endocytotic event. Complexes of pRSVL with free pL prepared with the same conditions contain similar doughnut structures, and there are examples in the literature of the formation of similar condensed complexes of lambda-DNA with polylysine at high salt (1M) concentration (Laemmli).

In a standard gene delivery experiment at the optimized conditions each DNA plasmid doughnut contains approximately 120 transferrin molecules. We could show that as we partially replace the TfpL conjugates with polylysine, transferrinfection efficiency is maintained (or even slightly increased) . However, we found that as we drop the transferrin content below 10-15 molecules per DNA molecule, the transferrinfection efficiency drops and falls essentially to zero if there is no TfpL in the DNA doughnuts.

It could be demonstrated that various other polycations can partially replace TfpL. There are two major instances where this "dilution trick" seems to result in a larger improvement: **1.** In cases where the DNA-binding group of the conjugate has lost its ability to condense DNA efficiently (e.g. transferrin conjugates with a synthetic protamine analogue: transfection efficiency is improved 20fold by pL addition), and **2.** In cases where the number of Tf-receptors is rather low and may be saturated by a surplus of Tf in the DNA complexes.

We conclude from our experiments that full condensation of plasmid DNA by polycations is one important factor in transfection efficiency. In recent experiments where part of a strongly condensing TfpL conjugate was replaced by the natural polycation histone H4,we found an additional improvement in transferrinfection. As EM data show, the role of histone H4 seems to be distinct from the condensation effect of other polycations.

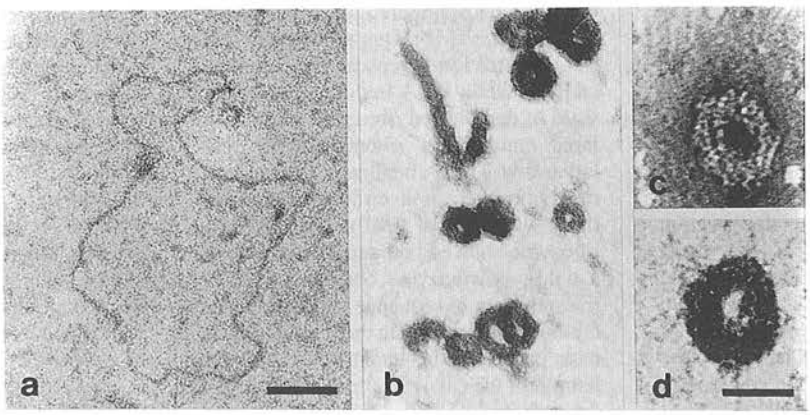

Fig.1. Complex of pRSVL plasmid-DNA with Tf -polylysine conjugates.
Electron microscopy of negatively stained (uranyl acetate) samples of plasmid DNA alone (**a**) , or 2 ug DNA complexed with 6 ug TfpL200C (**b-d**). Bar in a, representative for **a, b** is 100nm; bar in **d**, representative for **c** and **d** is 50nm. (In collaboration with Roland Foisner, University Vienna).

Further receptor mediated gene delivery systems

Many of the findings from the transferrinfection system may be useful for application to other receptor mediated gene delivery systems. Currently we are extending the polycation conjugate approach to different receptor ligands with other targeting specificities. Polylysine conjugates with monoclonal antibodies directed against CD4, with a recombinant HIV glycoprotein gp120 (Lasky et al., 1987). First experiments have shown that both the antiCD4 and the gp120 conjugates are able to mediate gene delivery to CD4 positive Hela and CHO cells.

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Oligonucleotide analogues: synthesis and modification of 2'-O-methyl-oligoribonucleotides

Berndt Oberhauser and Ernst Wagner

Modified antisense oligonucleotides (ONs) may become important tools for the study of gene regulation and inhibition of genes. Both the inefficient uptake of natural ONs into cells and their fast degradation by nucleases are substantial limitations. In our work, ON analogs with modified ribonucleotides and phosphorothioates have been investigated. In combined efforts with the groups of Doz. Ch. Noe (TU Vienna) and Prof. W. Pfleiderer (Universität Konstanz) we have synthesized phosphoramidites of 2' O-methylated ribonucleosides that were assembled to 2'-O-methyl-oligoribonucleotides (2'-methoxy-RNA); such analogues have been reported to be resistant to a variety of DNAses and RNAses (Sproat et al., 1989).In an in vitro inhibition assay of U7 mediated histone m-RNA processing M.Cotten (IMP) has shown that an antisense 2'-methoxy-RNA 19mer is 10 times more effective than a full-length antisense RNA and about 1000 times more effective than a 19mer antisense DNA. In order to assay the fate of 2'-methoxy-RNA *in vivo,* 19mer ONs were synthesized on a solid support bearing a 3'-aminolinker on the first (3') nucleotide. After deprotection and gel-purification the ONs were modified with fluoresceinisothiocyanate (FITC) and the fluorescent conjugate were HPLC-purified. Upon *transferrin*fection (Wagner et al., 1990) the uptake of the 2'-methoxy-RNA into cellular vesicles could be monitored by confocal microscopy (P. Steinlein, IMP) and quantified by fluorescence spectrometry of the lysed cells.

A lipophilic anchor provided to ONs may facilitate the uptake into cells (Shea et al., 1990),but such ONs may be partially trapped in membranes. To obtain a potentially bioreversible linkage of lipid to ONs , 2'-methoxy-RNA modified with an aminolinker at the 3' end was coupled to thiocholesterol via a SPDP disulfide bond. The conjugate was HPLC-purified and the cholesterol moiety was shown to be cleavable under mild reducing conditions with DTT.

To stabilize ribozymes of the hammerhead-type (Haselhoff et al., 1988; Cotten et al, 1989) against nucleolytic attack and to study the importance of the 2'-OH in catalysis and/or structure, several ribozymes of mixed ribo- and 2'-O-methylribo-sequences were synthesized using phosphoramidite chemistry (G.Schaffner, IMP). Due to the relatively stable protective groups on the commercially available RNA-building blocks, the yield of deprotected ribozyme containing large numbers of ribonucleotides was rather low. These chemically synthesized ribozymes were assayed for cleavage activity by B. Mandl (IMP). The 'all-ribo' ribozyme showed comparable activity to a wild-type ribozyme obtained by T7-transcription; a sequence containing 2'-methoxy-nucleotides in the regions which base pair with the target RNA and the hairpin of the catalytic site showed cleavage activity, although a bit less. The major limit seems to be the yield of complete deprotected material. On the other hand , 'full-methoxy' ribozymes could be synthesized in high yields and purity; however, replacing the single stranded conserved regions of the catalytic site with 2'-methoxy-nucleotides renders the ribozyme inactive.

In an attempt to identify an enzymatic method for synthesis of 2'-O-methyloligoribonucleotides, all four 2'- O-methylribonucleosidetriphosphates were synthesized chemically. The 2'-methylated ribonucleosides were 5'-phosphorylated with phosphorous oxychloride and coupled with pyrophosphate after activation with morpholine/DCC to yield the corresponding 2'-O-methyl NTP's. DNA primer extension with AMV reverse transcriptase using RNA-template in the presence of Mn^{2}+ and the four 2'-O-methyl NTP's gave only elongation up to 5 nucleotides. Complementation with dNTP's restored polymerase activity at dNTP concentrations above 500 nM (2'-O-methyl NTP's do not inhibit AMV reverse transcriptase). Transcription in buffers containing 1 M dNTP and 500 M 2'-methyl NTP therefore should partially incorporate 2'O-methyl nucleotides which gives the trancript a greater stability towards nucleolytic enzymes. This was shown in an preliminary experiment by a slight inhibition of degradation by micrococcal nuclease and exonuclease III. T7- as well as SP6 RNA-polymerase did not accept 2'-methyl NTP's as substrate in the presence and absence of cap-analogues. Furthermore there was a slight inhibition of T7 RNA-polymerase at 500 M 2'-methyl NTP concentration.

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Solid Phase Peptide Synthesis

Christian Plank and Ernst Wagner

Starting from the sequence of the natural protamine Salmine A1 (Warrant et al., 1978) an artificial protamine was synthesized. In this protamine, arginine residues which did not seem essential for binding of a single DNA strand were replaced by other neutral amino acids. This new protamine sequence was extended by a spacer of two glycines and provided with a cysteine at its C-terminus to be appropriate to coupling to other substances. The DNA-binding properties of this synthetic protamine proved to be comparable to natural protamine as shown by a band shift experiment with ^{32}P labelled l-HindIII DNA. This peptide can serve as the DNA-binding moiety in conjugates prepared for DNA transport experiments.

Two peptides with membrane-fusogenic properties have been synthesized. The sequence of one of these peptides was designed to form an alpha-helix at acidic pH with hydrophilic and hydrophobic side chains pointing to the opposite sides of the helix; this could provide the possibility of inserting channels into membranes. The other peptide is an analogue of the N-terminal 23 amino acids of the influenza virus haemagglutinin with a mutation of glycine-4 to glutamic acid. This N-terminal region is known to be involved in the haemagglutinin mediated fusion of viral and endosomal membranes (Stegmann et al., 1989). Both peptides were shown to disturb the integrity of liposomes as observed by the pH-dependent liberation of a fluorescent dye entrapped in the liposomes upon addition of peptide solutions to emulsions of liposomes. These peptides will be used to study the release of endocytosed material from endosomes .

Peptides for the purpose of generating antibodies

Peptides analogous to the N-terminal sequence of the Xenopus histone H1 variants H1A, H1B, H1C and to the C-terminal sequence of histone B4 were synthesized and coupled to soy bean trypsin inhibitor. With these conjugates it was possible to obtain highly specific antibodies against the histone variants which will be used in studies of histone expression during embryonic development (Harald Kandolf).

The Fmoc-derivative of e-acetylated lysine was prepared and used in the solid phase synthesis of a peptide analogous to the 18 N-terminal amino acids of acetylated calf histone H4. The peptide was used for the generation of antibodies which should help in the fractionation of chromatin on the basis of nucleosome acetylation (Adrian Bird).

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Analysis of the Genetic Principles of Tumor Suppression

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The work of our group is concerned with the localization, molecular cloning and characterization of genes which upon their functional loss contribute to tumorigenesis. Human cancer cells frequently show chromosomal aberrations resulting in partial monosomy and apparent loss of genetic material (e.g. Green 1988). These structural aberrations often affect regions that are specific for a given type of cancer. As a general hypothesis the deletions are suggested to affect genetic information whose functional presence is required to prevent the emergence of the tumor phenotype. Often this genetic information is referred to as "tumor suppressor genes", "recessive oncogenes" or "antioncogenes".

Allelic loss of DNA sequences: locating putative tumor preventing genes

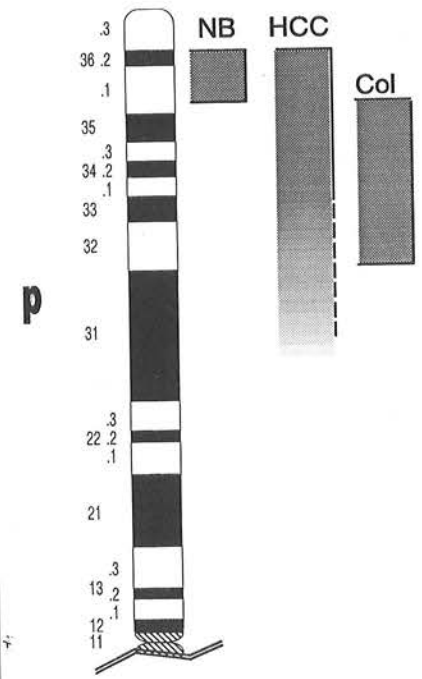
a) Neuroblastoma

In human neuroblastomas, at least 70% of tumors display deletions and/or translocations in the distal part of the short arm of chromosome 1 (see Heim and Mitelmann 1987). In pursuit of a molecular analysis of this region we have generated a microclone library from microdissected 1p35-1pter chromosome fragments (Martinsson et al. 1989). Probes of this library have been mapped within the region of interest by Southern mapping on a panel of human X mouse hybrids containing different parts of the human chromosome 1 as the only human material. In addition chromosomal in situ hybridization has been performed for the fine mapping of a subset of probes. Microcloned probes thus localized and detecting restriction fragment length polymorphisms (RFLP) were then employed to detect allelic loss in DNA of neuroblastoma tumors as compared to DNA of matching normal tissue. We observed a loss of alleles in 90% of the tumors analyzed (Weith et al. 1989). The allelic deletions consistently involved material within the chromosomal bands 1p36.12-p36.2. This region roughly comprises 7-8 Mbp (Megabase pairs) of DNA. The high incidence of allelic deletions in neuroblastoma cells suggests genetic information involved in tumorigenesis to reside within this region.

b) Hepatocellular carcinomas

in collaboration with D. Simon (Wistar Institute, Philadelphia).

We recently extended our loss of heterozygosity studies to other cancers



Tumor-specific consensus deletions on chromosome 1p in three different cancer types. Consistently deleted regions are shown as shaded areas with silod horizontal bars where borders could be defined.

NB: neuroblastoma,

HCC: hepatocellular carcinomas,

Col: colon carcinomas

Using our 1p-specific probes we detected loss of DNA sequences in a significant number (five out of seven) of hepatoblastomas and hepatocellular carcinomas (Simon et al. 1990). In most cases the allelic deletions observed in liver tumors covered considerable areas of the distal 1p arm, the distal part of them matching the neuroblastoma consensus deletion. However, a proximal border for a consensus deletion in hepatocellular carcinomas could not yet be defined using our probes.

c) Colorectal cancers

in collaboration with M. Schwab (DKFZ, Heidelberg).

A comparable study of approx. 60 colorectal tumors (Leister et al. 1990) also revealed allelic deletions within the chromosome 1p arm, though occuring at a lower frequency (48% of the tumors analyzed). In this cancer system, however, a consensus deletion did not match the neuroblastoma deletions but rather maps more proximal (encompassing band 1p35).

From our loss of heterozygosity analyses it became obvious that at least the consensus deletions of neuroblastomas and of colon carcinomas do not overlap (**see figure**). We therefore suggest that more than one gene involved in tumorigenesis may be located in the chromosome 1p arm.

Molecular analysis of genetic elements in the neuroblastoma consensus deletion

At present our information on tumor-specific allelic deletions is most detailed for neuroblastomas. We therefore set out first to identify a neuroblastoma-associated gene from the 1p36.12-p36.2 region. The approach to molecularly clone this gene involves

- establishing a long range map and identifying CpG-rich islands using pulsed field gel electrophoresis,
- identifying DNA sequences being conserved in evolution and thus probably being functionally important,
- screening CpG island sequences and conserved DNA fragments for transcribed elements by Northern hybridization.

So far we have isolated two CpG islands from the region of interest. Another four will be cloned shortly by short-distance Cosmid walking. A DNA sequence from one of the cloned islands was found to encode for an RNA which is ubiquitously transcribed in tumor and non-tumorigenic tissues.

Using Southern hybridization of microcloned probes to interspecies blots ("zoo blots") we so far identified four DNA fragments conserved in various mammalian species, another two being homologous to sequences in all vertebrates tested, and one displaying homologies also in yeast. Northern hybridization of these probes is in progress.

Identification of balanced translocations in tumors: spotting the putative tumor-preventing gene

The successful isolation of a tumor-associated gene from the neuroblastoma consensus deletion necessarily involves the analysis of many genes within this region. In order to avoid this time-consuming approach we attempt to identify a tumor where the only significant genetic rearrangement is a balanced translocation in the distal 1p region, the breakpoint thus indicating the position of the gene of interest. We are trying to identify such translocations cytogenetically by specifically "painting" the distal 1p arm with a pool of fluorescence labeled DNA probes. Using our entire 1p35-pter microclone library as a labeled probe we expect to mark the region of interest, balanced translocations thus being readily visible as splittings of the hybridization signal. Initial experiments using microclone libraries as probes for "chromosomal in situ suppression hybridization (CISS)" were performed successfully in collaboration with T. Cremer and P. Lichter, Heidelberg (Lengauer et al. 1990).

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Nuclear oncoproteins affecting hematopoietic cell differentiation

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Introduction

The cellular homologues of a number of oncogenes (proto-oncogenes) have proven to constitute important components of an intimate network which governs cell growth, proliferation and differentiation. Their genetic alteration, over- and/or ectopic expression often led them to acquire oncogenic potential, presumably by interfering with or by inappropriately mimicking normal cellular signal transduction pathways.

As described in the last years research report our interest has focused on the biology of the v-erbA oncoprotein and the way it contributes to leukemogenesis. This includes (1) the analysis of the genetic alterations which turns the normal thyroid hormone receptor c-erbA into an oncoprotein and (2) studies which genes might be affected by erbA and by what mechanism this occurs (in collaboration with the group of Hartmut Beug, IMP, Vienna).

Recently we have initiated experiments to investigate if and how other nuclear oncoproteins, nuclear hormone receptors and other nuclear proteins involved in gene regulation might alter the differentiation program of hematopoietic cells and thus contribute to leukemogenesis if inappropriately or overexpressed in such cells. Therefore preliminary experiments have been performed to study certain aspects of myc, myb, fos, jun and rel oncogene function affecting hematopoietic cell differentiation.

In addition, we continued our studies of using transferrin-polycation conjugates to introduce DNA into cells via the transferrin receptor by receptor-mediated endocytosis (in collaboration with Ernst Wagner, Matt Cotten, Max Birnstiel and Hartmut Beug, IMP, Vienna).

I. Molecular biology of the v-erbA oncogene

1. *Mechanism of action of the v-erbA oncoprotein in erythroleukemia*

v-erbA encodes a highly mutated version of the thyroid hormone(T3/T4) receptor c-erbA (type a: Sap et al., 1986; Weinberger et al., 1986). However, owing to multiple point mutations and a small C-terminal deletion, the v-erbA oncoprotein lost the ability to bind T3 but still binds to DNA in a sequence-specific fashion (see Zenke et al., 1990 for references).

v-erbA was initially identified as one of the two oncogenes of the avian erythroblastosis virus (AEV), a retrovirus causing sarcomas and an acute fatal erythro-leukemia in virus-infected chickens (for a review see Graf and Beug, 1983). Previous studies demonstrated that v-erbA exerts its oncogenic potential in erythroid cells by efficiently blocking terminal differentiation of both normal and transformed erythroid precursor cells (termed erythroblasts) into normal erythrocytes (Zenke et al., 1988, 1990; Schröder et al., 1990 and references therein). In addition, v-erbA abrogates the complex growth requirements of such transformed erythroblasts in culture (Beug et al.,1985; Kahn et al., 1986). A growing body of evidence suggests that v-erbA causes its specific effects in these cells by interfering with transcription of erythroid-specific genes, expression of which is upregulated during and most probably required for normal erythrocyte differentiation (Zenke et al.,1988, 1990, Knight et al.,1988). Genes, the expression of which is suppressed by v-erbA, encode the erythrocyte-specific anion transporter band3, the erythroid-specific carbonic anhydrase II (CAII) and the enzyme d-aminolevulinate synthase (ALA-S, which catalyses the first and rate-limiting step in heme biosynthesis). Recently it was found that phosphorylation of v-erbA at two specific serine residues (Ser16 and Ser17; Sap et al., 1986) is required for its function as an oncoprotein (Glineur et al., 1990).

We have subsequently investigated the possible role of the normal thyroid hormone receptor c-erbA in erythroid cell transformation and/or differentiation, utilizing suitable c-erbA-expressing retroviruses. We found that in erythroid cells expressing high levels of exogeneous c-erbA, erythrocyte differentiation is re-

pressed in the absence of thyroid hormone (T3) but activated after addition of T3 (Zenke et al.,1990). In these cells, transcription of those erythrocyte-specific genes which are repressed by v-erbA (CAII, band3, ALA-S) was regulated in a hormone-responsive fashion (repressed in the absence and induced in the presence of T3). This suggests that the v-erbA oncoprotein has lost one type of normal receptor function (i.e. to regulate transcription in response to hormone), but constitutively displays another function: it represses transcription in the absence of T3. The region responsible for the loss of hormone-dependent regulator activity has been mapped to a short deletion present at the very C-terminus of v-erbA, encompassing in c-erbA a cluster of highly conserved amino acid residues with the potential to form an amphipatic alpha-helix (Zenke et al.,1990).

2. *Identification of erbA binding sites in the erythrocyte-specific carbonic anhydrase II (CAII) gene.*

To understand the mechanism of action of the erbA proteins on a molecular basis efforts have been made to identify a direct target gene of erbA action. Several lines of evidence do suggest that CAII is such a direct target gene: The induction of CAII transcription by T3 in erythroid cells expressing c-erbA or chimeric v-/c-erbA proteins is both rapid and does not require protein biosynthesis (as evidenced by its insensitivity to cycloheximide treatment; Disela et al., manuscript in preparation). An in vitro DNAseI protection assay revealed multiple v-erbA binding sites in the CAII promoter. The analysis of one of these sites showed that it also bound the normal thyroid hormone receptor c-erbA. Furthermore, this erbA binding site constitutes a bona fide thyroid hormone-responsive element (T3RE) since it conferred thyroid hormone responsiveness to a heterologous promoter in transient expression assays. Studies to reveal the biological activity of the other erbA binding sites identified in the CAII promoter are currently in progress. In addition, it will be interesting to determine whether erbA protein phosphorylation (Glineur et al., 1990) does affect its ability to interact with such specific DNA sequences and to repress and/or stimulate transcription from luciferase- or CAT-encoding reporter constructs in transient transcription assays.

II. Transferrin-polycation conjugates as carriers for DNA uptake into cells via receptor-mediated endocytosis.

(in collaboration with Ernst Wagner, Matt Cotten, Max Birnstiel and Hartmut Beug, IMP, Vienna)

Last year we reported that transferrin-polylysine (TfpL) and transferrin-protamine (Tfprot) conjugates can be used to efficiently introduce DNA into hematopoietic cells, a method termed *transferrinfection* (Wagner et al.,1990; Zenke et al.,1990; Cotten et al.,1990). The transferrin-polycation-DNA complexes are taken up by the cells via the transferrin receptor by receptor mediated endocytosis. Further experiments showed that following uptake, expression of the DNA is transient. However, repeated administration of the TfpL-DNA-complexes ensures efficient expression of e.g. a *transferrinfected* luciferase-encoding reporter plasmid over an extended period of time (**4 days; see Figure**). No cytotoxic effects have been observed during this time. Recently we have also used transferrinfection of retrovirus vector DNA carrying the neomycine-resistance gene to generate stable transformants (see Figure).

To optimize the efficiency of *transferrinfection* various modified transferrin-polycation conjugates have been tested:

(1) poly-D-lysine instead of poly-L-lysine since poly-D-lysine (a polymere of an artificial amino acid which does not occur in nature) might be less susceptible to enzymatic degradation.

(2) transferrin-polyarginine (TfpArg) as compared to TfpL and Tfprot.

(3) thioether as compared to disulfide linkage between the transferrin (Tf) and polylysine moiety of the TfpL conjugate.

(4) various chain lengths of the pL (90 to 450 amino acid residues) in the TfpL conjugate.

Several lines of evidence suggested to us that the TfpL-DNA complexes travel through and/or accumulate in endosomal- or phagosomal-like compartments within the cell. Because of the low pH values present in these vesicles we argued that a pH-sensitive linkage between the Tf and pL moiety of the TfpL-DNA complex might facilitate release of the pL-DNA complex and increase DNA expression. Consequently TfpL-conjugates containing a linkage through an acid-sensitive ketal function were synthesized and their sensitivity to low pH values confirmed in vitro (Ernst Wagner).

Of all modified TfpL conjugates tested only those with an increased pL chain length significantly improved the gene transfer and expression efficiency of an luciferase-encoding reporter gene in transient expression assays.

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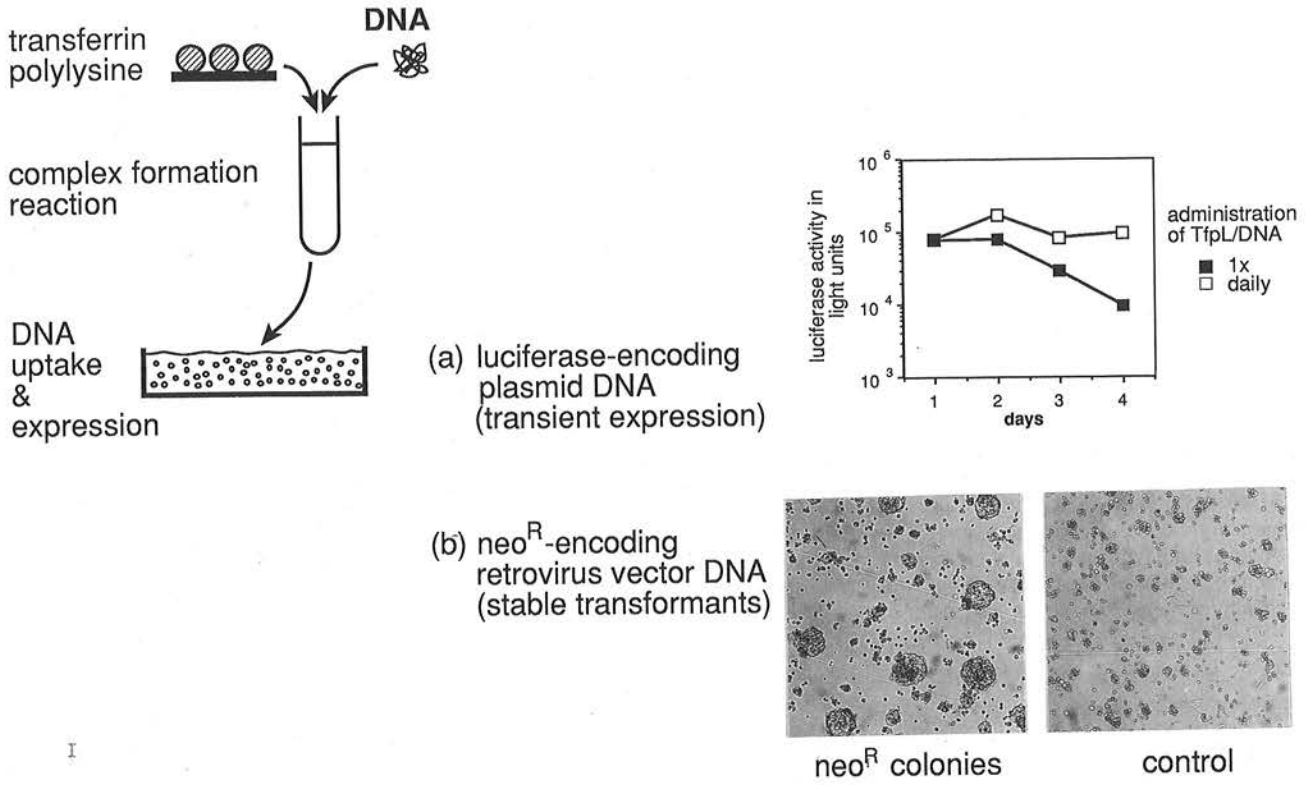

Long-term expression of the DNA transferrinfected into erythroid cells is achieved by (a) repeated administration of the transferrin-polylysine-DNA complexes or (b) by stable integration of the DNA into the cellular chromosome.

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Animal House

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Gerhard JURITSCH	Technician
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The animal house provides technical support to the scientists and at present holds various in and outbred as well as hybrid strains of mice, random-bred rabbits, out-bred chickens and random-bred Xenopus laevis. Current production of animals bred in-house is as follows: approx. 250 mice/week; 10 chicken embryos/week and 12 chickens/week (in conjunction with Bender). We have recently started to rear our own Xenopus laevis toads, but due to the long maturation time it is still necessary to purchase this species from external suppliers. Rabbits are also purchased from external suppliers for economic reasons.

The policy of the Animal House is to provide the highest possible technical and scientific support with respect to animal experimental studies. It is essential to these studies that animals are bred and maintained to a defined genotype with minimal disease, since the factors may affect the outcome of experimental results.

Essentially, the animals listed above service the following studies: various mouse projects (see reports of Erwin Wagner and Denise Barlow); chicken leukemia studies (see reports of Hartmut Beug and Martin Zenke); and Xenopus laevis studies (see report of Max Birnstiel).

Since January 1990, the technical procedures offered by the Animal House, in addition to those already established, such as collection of rabbit and chicken blood and administration of hormones to mice, have been increased to include tumour implantations, collection of blood by cardiac puncture in mice and chicks, intravenous injections of mice and chicks, hysterectomy and subsequent resuscitation of potential transgenic and chimaeric mice, collection of tissue such as skin and muscle for DNA analysis by the scientist and breeding programs to give designated number of animals requested, thereby avoiding overproduction.

It is hoped that the technical procedures performed by the Animal House staff will increase still further during the next year.

Computer Group (Protein Modelling)

Anton BEYER	Scientist
Georg CASARI	PhD-student
Aron BAUER	Diploma student
Eva SILHAVY	Diploma student

Introduction

We are mainly concerned with protein structure calculations and the relationship between amino acid sequence and tertiary fold of a protein. Currently there are three students of the University of Vienna working in my group.

There are three main topics we are currently investigating.

1. The first approach is based on mathematical models using empiricial potential functions. The methods used are energy minimization, molecular dynamics calculations and simulated annealing.
2. The second approach uses purely empirical data from known crystalstructures to obtain a potential of mean force using a newly developed method. This potential of mean force can then be used to predict structural preferences of short sequences. This work is done in close collaboration between Georg Casari and the group of Manfred Sippl of the University of Salzburg.
3. The third main line of our work is concerned with structure prediction of homologous proteins. Secondary structure prediction methods, sequence alignment, structural data from known proteins and computer graphics methods are used to model the structure of the unknown protein. One aspect of this work is the development of a relational database for protein structures where experimental data from a variety of different sources can be included. This information can then be used easily in protein modelling projects.

A new Approach to Protein Structure Prediction Calculations on Proteins: Energy potentials

Georg Casari

All calculations on protein structures use some kind of energy potentials for the proteins to evaluate the probability of a certain fold. The most widely used potentials are based upon modelling the amino acids atom by atom. These potentials have failed to give a useful means to evaluate or predict a structure of a protein (1).

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 looked extremely promising (see (2)). In further tests applying the method we could identify the native fold for a given sequence among a large set of alternative structures with an extremely high rate (3).

At present we are elucidating the physical and physical-chemical background of these potentials and try to reduce the inaccuracies that are a consequence of our limited set of known structures.

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Modelling of Homologous Proteins: Rhinovirus 2A Protease

Georg Casari

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. Secondary 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 m odel 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.

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Conformatibnal Calculations on PHOSPHOLIPASE A2

Aron Bauer

Based on the experience that rather subtle changes in local secondary and tertiary structure go along with single site mutations or single amino acid exchanges in proteins, modelling of these systems is usually done by means of a minimum perturbation approach (1). In fact there are examples for amino acid exchanges that do hardly alter the functional properties of the protein but distort local structure at a high degree. Classical methods of structure prediction would surely fail to foresee these changes (2). The known x-ray structures of Phospholipase A2 (bovine and porcine) provide such an example (3). We are investigating the possibility to predict that kind of vast local rearrangements of a polypeptide chain using Molecular Dynamics in combination with Simulated Annealing, a technique that should suffice to find more global minima than simple energy minimization. The most striking problem is still the enormous amount of computing time these methods require. We are trying to reduce the simulated system in size by neglecting the invariate part of the molecule and applying constraints and restraints that simulate the effects of that part on the remaining atoms. These calculations are done in vacuo as well as in aqueous solution.

Simulated Annealing goes along with completely rearrangement of the interesting part of the polypeptide chain. Structures that are roughly similiar to the desired structure can be observed, but it seems hard to decide which of the found structures is closest to reality. The potential functions used do not provide a criterion for this kind of decision.

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Outlook:

The combination of different the mathematical methods and their use for protein structure prediciton is the main topic of our work.

The following projects are ongoing efforts along these lines.:

A. Beyer

Design and implementation of a structural database for proteins.

Georg Casari, in close collaboration with M. Sippl from the University of Salzburg.

Using the concept of a potential of mean force for structural investigations on proteins.

A. Bauer

Structural Calculation on Phospholipase A 2 using molecular dynamcis and simulated annealing.

E. Silhavy

Calculations on several point mutations of Lysozyme to explore the influence of certain residues on the structure of the protrein. The results of these calculations are carefully compared to X -ray structural data and are used to test the predictive power of these calculations.

G. Casari in collaboration with E. Kuechler, University of Vienna and G. Sommergruber, Bender & Co, Vienna.

Modelling of the human rhinovirus 2A protease.

G. Casari in collaboration with M. Nicklin, IMP Vienna

Modelling studies on the Leucine Zipper of fos and jun.

Publications 1990

A. Beyer, P. Schuster

Internal Dynamics of Flexible Molecules: Cyclohexane: Monatshefte f. Chem. 1212, 2339/349 (1990)

H. Schreiber

Simulated Annealing und Molekulardynamik zur Konformationssuche von Peptiden.

Moeglichkeiten und Grenzen am Beispiel des Heptapeptides Deltorphin: Diplomarbeit, Univ,. Wien ,September 1990

M. Hendlich, P. Lackner, S. Weitckus, H. Floeckner, R. Froschauer, K. Gottsbacher, G. Casari, M. Sippl

Identification of Native Protein Folds Amongst a Large Number of Incorrect Models

J. Mol. Biol. Vol. 215 in press

Workshop organized by G. Casari

Protein Structure and Dynamics in Theory and Experiments

September 4/7 1990, Spitz /Donau

Service-Department: DNA-Sequencing and oligonucleotide synthesis

Gotthold SCHAFFNER Scientist
Sissy AIGNER Technician
Ivan BOTTO Technician
Robert KURZBAUER Technician

DNA sequencing and the synthesis and purification of oligo-nucleotides are the most popular services of the I.M.P. Service Department. They consume the bulk of our working time. In addition we provide, with the help of the animal house staff, rabbit antisera to isolated proteins and various small oligopeptides. The titers of these sera are regularly tested. We also serve as an inhouse depository of cloning vectors, primers and transformation competent bacterial *E.coli* strains, as well as providing selected reagents (e.g. moleclar weight markers, enzymes etc.).

Oligonucleotide Synthesis

The regular use of the PCR technique resulted in an increased demand for primers of about 30 nucleotides in length. We synthesized greater than 1000 oligonucleotide on a single machine, a three column APPLIED BIOSYSTEMS 380 B synthesizer, using the phosphoramidite chemistry. The PHARMACIA GENEASSEMBLER was mainly used for test synthesis (e.g. 2'-methoxy RNA).

Short oligonucleotides <25 nucleotides (e.g. sequencing primers or PCR primers) were used directly for the reactions after only an ethanol precipitation for purification. Oligonucleotides >25 nucleotides were isolated by HPLC or on polyacrylamide gels followed by purification over SEPPACK C18 reversed phase columns.

The synthesis of RNA- and especially of 2'-methoxy-RNA oligo-nucleotides has become more efficient because of the improved quality of the monomers prepared by Dr.Noe's group at the Technical University of Vienna and a new method for phosphorylation for the 2'-methoxy-ribonucleotides introduced by Berndt Oberhauser at the I.M.P. The synthesis of fully deprotected oligoribonucleotides is still a problem, because the commercially available monomers contain the much more stable benzoyl- and isobutyryl-residues on the base instead of the more readily cleaved phenoxyacetyl-group. We found that an oligoribonucleotide isolated from a band of a polyacrylamide gel and then analyzed by HPLC on a reversed phase column contains many molecules possesing protecting groups still on it. Nevertheless we successfully synthesized and isolated biologically active ribozymes as homoribo- or as hetero-ribooligomers containing 2'-methoxy- ribonucleotides outside the catalytically active domains.

Sequencing

Greater than 2500 sequencing reactions with crude double stranded DNA were made during 1990. Many of them were performed to determine clones with plasmids containing the oligonucleotide of the correct sequence. DNA prepared from "minipreps" by alkaline lysis or by the "boiling prep procedure" was directly used for sequencing. Alkaline denaturation at 85°C and the addition of manganese sometimes helps to overcome template problems. With optimized conditions in the SEQUENASE (USB) protocol using T7 DNA polymerase (PHARMACIA) the total amount of double stranded DNA seems to be the limiting factor of the readable sequence on a gel.

In addition to sequencing cloned oligonucleotide constructs, we have analyzed more than 30 kbp of unknown genes. For these reactions we are using DNA isolated from CsCl-gradients and again the double strand sequencing protocols with primers synthesized for those DNAs ("primer walking").

Recently Robert Kurzbauer has "trained" the BIOMEK 1000 (Beckman) robot with the single tip head to produce sequencing reactions of comparable quality to those we do by hand. The results are very promising.

Publications

Cotten, M., Gick, O., Vasserot, A., Schaffner, G. and Birnstiel, M. L.(1988) EMBO J. 7, 801—808.

The PR-Office

Martina STEINHARDT Scientific PR-speaker

The PR-Office has to fulfil a variety of tasks required by the public. Run by Martina Steinhardt, a cell biologist, it can be called the "Information Service Station" of the I.M.P. In 1990 for example, a great need for information about melanoma treatment arose, due to a talk by Prof. Steven Rosenberg at the 3rd International I.M.P. conference about a newly adopted method to treat terminal stage melanomas. We feel that whenever it seems appropriate, we should at least provide a little help for people with cancer, and in doing so we can narrow the large gap between medical research and patient.

The activities of the PR-Office in the year 1990 were as follows:

Interviews and discussions with journalists

For the last years the public interest in the so-called "going-on's behind the walls of scientific institutes" has more and more developed. Due to the nuclear catastrophe at Chernobyl and possible future accidents of this kind, environmental pollution and the exponential growth of knowledge, the laypeople have become afraid of being overrun by science. Malaise has also been triggered by Science fiction literature like Aldous Huxley's "Brave New World" or "The Day After". The result was an increase in journalists writing about science and scientific progress. Particularly with the controversy about gene technology, journalists seek proper information to pass it on. In 1990, we therefore organized a press conference in the framework of the third I.M.P. conference on gene transfer and alteration in Biology and Medicine. Later, a Journalists' seminar on the genetics of cancer was held together with Bender and PHARMIG followed by a very positive reaction by the attending journalists. In addition, a discussion forum with scientists, PR-speakers from other research companies and journalists was founded. After the first general meeting we talked about possible laws concerning gene manipulation in Man. This group will meet regularly with different topics for discussion. The next meeting will be in February concerning agricultural aspects of gene technology.

Apart from these activities, interviews were given not only to journalists of the local papers but also on radio and TV. The most positive feedback we got from the TV-interview in "Wissen Aktuell" about the successful melanoma treatment by Prof. Dr. Steven A. Rosenberg.

Visiting groups

In 1990, we had 21 visiting groups. 7 of them were high school students and teachers, the latter to update their knowledge of molecular genetics. It was also very encouraging to have had people from the Socialist's Party, the Green Party and the "Gen-Ethical-Network" (Gen-Ethisches Netzwerk) for constructive and informative talks. We also arranged an Open Day for the public and invited district officers and our neighbourhood for discussions.

Representation of the I.M.P. in the Public

According to the hot discussions about gene technology not only in Austria, but all over the world, it is expected that as an international research institute the I.M.P. be represented at public discussions, meetings and TV sessions if requested. Several public seminars about gene technology and its consequences were attended:

- podium discussion "GENiale Welt", a women's conference of the Austrian Socialistic Party concerning gene technology and its consequences to women (in Salzburg);
- podium discussion: "Gentechnik – Was wissen und was wollen wir?" ("Gene technology – what do we know, and what do we want?"), Renner-Institute, Vienna;
- Seminar on genetic engineering at the Universität für Bodenkultur, Vienna;
- regular PHARMIG-PR-meetings, organized by the chairwoman of the PHARMIG, Mrs. Mayrhofer;
- second discussion "GENiale Welt" by the Socialistic Youth of Austria (St. Pölten);
- Seminar "Science and Media" in Frankfurt (Germany);

- discussion about PR and universities (Raiffeisenhaus, Vienna);
- Press conference "Laws concerning gene technology" at the Länderbank (Vienna).

Last, but not least, it should be mentioned, that I was invited to give two talks on a more advanced scientific level: one at the Institute of General and Molecular Biology, Masaryk University, Brno, and one at the Hospital Krankenhaus der Barmherzigen Brüder.

One of the future goals for 1991 will be a monthly press Info-paper with the latest I.M.P. news.

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IMP SEMINAR LIST 1990

11.01.90	**G. WICK** (Univ. Innsbruck) "Animal models for human autoimmune diseases"
18.01.90	**WARREN ALEXANDER** (Walter & Eliza Hall Inst., Melbourne)
25.01.90	**JOHN JENKINS** (Marie Curie, Oxted) "p53 links DNA replication and the eukaryotic cell cycle"
01. 02.90	**MARTIN VINGRON** (EMBL) "Protein sequence comparison: Improved methods and reliability of results"
08. 02.90	**TOMAS LINDAHL** (ICRF, Clare Hall Labs.) "Biochemical deficiencies in human chromosome breakage syndromes"
15.02.90	**HERBERT JAECKLE** (Munich) "How to make stripes in an embryo?"
22.02.90	**GARETH GRIFFITHS** (EMBL) "Endocytosis in animal cells"
Tues., 12.30 pm	
27. 02.90	**RODNEY ROTHSTEIN** (Univ. of Columbia) "Mutation of a novel eukaryotic topoisomerase alters genetic recombination"
Tuesday, 5.30 pm	
06.03.90	**JOHN HEATH** (Oxford University) "Growth and differentiation factors of embryonic stem cells"
08. 03.90	**MANFRED SCHWEIGER** (Innsbruck University) "Protein modification in human DNA repair"
15.03.90	**JOE JIRICNY** (Basel) "5-Methylcytosine: a link between gene expression and mismatch repair in higher eukaryotes?"
22.03.90	**IAN JACKSON** (MRC, Edinburgh) "Mouse developmental genetics: Looking at old mutations and making new ones"
29.03.90	**AARON MITCHELL** (Columbia University) "Positive control of yeast meiotic genes by a protein kinase homolog and its targets"
Friday, 12.00 noon	
30.03.90	**BRECK BYERS** (Washington University) "Yeast spindle pole regulation"
05.04.90	**RICCARDO CORTESE** (EMBL, Heidelberg) "Transcription factors and the liver phenotype"
Monday, 2.00 pm	
09.04.90	**ROCHELLE EASTON ESPOSITO** (University of Chicago) Regulation of meiotic genes controlling recombination and chromosome segregation"
19.04.90	**WOLFRAM OSTERTAG** (Hamburg) "LTR insertion mutagenesis as a tool to study normal and abnormal proliferation in hematopoietic cells"

26.04.90	**PETER GOODFELLOW** (ICRF, London) "Sex determination in man"
02.05.90	**JEREMY GREEN** (NIMR, London) "Specification of cell types in Xenopus embryos by a morphogen gradient"
03. 05.90	**HARALD ZUR HAUSEN** (DKFZ, Heidelberg) "Papillomaviruses in human cancer"
Wed. 12.30 pm **09.05.90**	**MAGNUS PFAHL** (La Jolla Cancer Research Foundation) "Repressor and activator functions of nuclear receptors"
10.05.90	**AZIM SURANI** (AFRC, Cambridge) "Genomic imprinting: Epigenetic control of development and genetic disorders"
Tuesday, 5.30 pm **22.05.90**	**MANFRED SIPPL** (Univ. Salzburg) "Protein folding by potentials of mean force"
Friday, 12.30 noon **01.06.90**	**JEFF SCHELL** (MPI, Cologne) "Genes involved in the control of differentiation in plants"
Friday, 4.00 pm **01.06.90**	**GERRY FINK** (The Whitehead Inst.) Some unexpected aspects of gene regulation in yeast
07.06.90	**PHIL INGHAM** (ICRF, London) "Genetic analysis of cell patterning in the Drosophila embryo"
Monday, 4.00 pm **18.06.90**	**ULRICH RÜTHER** (EMBL, Heidelberg) "Probing development with molecularly defined mouse mutants"
20.06.90	**WOLFGANG PFLEIDERER** (Univ. Konstanz) "The oligoribonucleotide problem" (Chemical synthesis of RNA molecules)
21.06.90	**WALTER GEHRING** (Biozentrum, Basel) "Exploring the Homeobox"
28.06.90	**ANDREI MIRZABEKOV** (Univ. of Moscow) "New approaches to sequencing DNA and proteins along DNA Organization of active chromatin."
05.07.90	**FRANCESCO BLASI** (Univ. of Copenhagen) "Molecular biology of urokinase plasminogen activator"
impromptu, 12.30 pm **06.07.90**	**DIMITRIJ PLACHOV** (MPI, Göttingen) "PAX 8 murine paired box-containing gene: its structure and expression during embryogenesis"
impromptu, 12.30 pm **10.07.90**	**WALTER KOLCH** (NIH, Frederick) Functional analysis of Raf-1 by inhibition studies"
12.07.90	**ERWIN RÜDE** (Mainz) "Accessory signals in antigen-dependent and antigen-independent stimulation of CD4+ murine T-cells"

19.07.90	**TED WEINERT** (Univ. of Arizona) "Checkpoints: Coordination of mitosis and DNA replication in the cell cycle"
impromptu, 2.00 pm **25.07.90**	**BASTIAN HENGERER** (MPI, München) "Involvement of c-fos in NHF regulation"
impromptu, 12.30 pm **02.08.90**	**KURT STOECKLI** (MPI, München) "Molecular cloning, functional expression and distribution of rat cillary neurotrophic factor (CNTF) in the adult and developing nervous system"
02.08.90	**JOHN KILMARTIN** (MRC Cambridge) "Yeast spindle pole body components and their possible roles in mitosis"
08.08.90	**CHRISTOPH BRUNNER** (EMBL) Processing of ribosomal rRNAs in Saccharomyces cerevisiae"
impromptu, Monday, 5.30 pm **20.08.90**	**CHRISTINE GUTHRIE** (UCSF) "snRNPs and splicing: Genetic approaches in yeast"
Tuesday, 5.30 pm **21.08.90**	**HAROLD VARMUS** (UCSF) "Retroviral Oncogenes"
impromptu **23.08.90**	**DAVID WESTAWAY** (UCSF) "Genetics and transgenetics of scrapie and related diseases"
30.08.90	**JEAN-MARIE BLANCHARD** (Montpellier) "Some new aspects of c-fos gene regulation"
06.09.90	**DAVID LANE** (ICRF, Clare Hall Labs) "Mutation of the p53 gene, a common step in most Human Cancers"
impromptu, 2.00 pm **07.09.90**	**FRANCIS BARANY** (New York) Thermus aquaticus DNA recognition proteins, and their use for detection of genetic diseases"
20.09.90	**BERND GRONER** (FMI, Basel) "Hormones and oncogenes regulate growth and gene expression in mammary epithelial cells"
27.09.90	**JIM SMITH** (NIMR, London) "Activins and thresholds in the formation of embryonic mesoderm"
Monday, 12.30 pm **01.10.90**	**ROGER CHALKLEY** (Vanderbilt Univ.) The role of chromatin structure in the developmental and hormonal control of the PEPCK gene"
Monday, 5.30 pm **08.10.90**	**TAKASHI KEI KISHIMOTO** (Boehringer Ridgefield) "Role of LECCAMS in neutrophil and lymphocyte interaction with endothelium"

Tuesday, 5.30 pm **09.10.90**	**B.J. BORMANN** (Boehringer Ridgefield) "Transmembrane domain association in signal transduction"
Wednesday, 5.30 pm **10.10.90**	**F. SOLYMOSY** (Szeged, Hungary) "Uridylate-rich small nuclear RNAs (UsnRNAs), their genes and pseudogenes"
Wednesday, 12.30 **10.10.90**	**ROGER FLEISCHMAN** (University of Texas) "Regulation of stem cell proliferation by the c-kit proto-oncogene and isolated stromal cells"
11.10.90	**KLAUS KRATOCHWIL** (Salzburg) "The Mov13 insertion mutation: Its relevance for the regulation of collagen genes and for provirus activation"
Tuesday, 5.30 pm **23.10.90**	**ANDRE SENTENAC** (CEA, Gif-sur-Yvette) "Class B factor involved in the transcription of yeast U6 gene by RNA polymerase C"
Wednesday, 12.30 pm **24.10.90**	**MARION FUNG** (Duke Univ.Med.Center) "The human interleukin-2 receptor: structure, expression, and mechanism of signal transduction"
Impromptu **30.10.90**	**GENNARO D'URSO** (Fred Hutch., Seattle) "Cell cycle regulation of DNA replication by the p34CDC2 protein kinase"
08.11.90	**ED HURT** (EMBL, Heidelberg) "Yeast as a eukaryotic model system to study nuclear pore and nucleolar structure and function"
15.11.90	**PATRICK CHARNAY** (CNRS, Paris) "Mouse zinc finger genes potentially involved in the regulation of cell proliferation and development"
22.11.90	**JIM NEIL** (Beatson Inst.Glasgow) "Molecular mechanisms of leukaemogenesis by a C-type retrovirus"
29.11.90	**KAI SIMMONS** (EMBL, Heidelberg) "Sorting of surface glycoproteins in polarized cells"
06.12.90	**VINCENZO SORRENTINO** (EMBL, Heidelberg) "From growth arrest to growth suppression"
13.12.90	**DAVID ISH-HOROWITZ** (ICRF, Oxford) "Helix-loop transcription factors in Drosophila segmentation and sex-determination"
Impromptu, 12.30 pm **19.12.90**	**MARGIT BURMEISTER** (UCSF) "Mapping chromosome 21 to understand Downs Syndrome and Alzheimers Disease"

RESEARCH INSTITUTE OF MOLECULAR PATHOLOGY (I.M.P.)
3rd INTERNATIONAL CONFERENCE ON

"GENETIC TRANSFER AND ALTERATION IN BIOLOGY AND MEDICINE"

organised by: Ardian Bird, Max L. Birnstiel and Erwin Wagner (Vienna).
Marc Van Montago (Gent) an Richard Mulligan (Cambridge, USA)
Conference Manager: Judith Nicholis, Tel: (0222) 792636-504

The conference will review genome mapping and sequencing, Inhibition of gene expression by antisense oligos and ribozymes, transgenic plants and applied plant molecular biology, transgenic mouse models for human diseases as well as approaches to gene therapy in man. The invited speakers include:

A. Berns (Amsterdam)
A. Bernstein (Toronto)
M. Birnstiel (Vienna)
N. Chua (New York)
H. Cooke (Edinburgh)
H. Donis-Keller (St. Louis)
R. Evans (La Jolla)
A. Gaterby (Wilmington)
W. Gilbert (Cambridge, USA)
D. Gierson (Nottingham)
D. Hartl (St. Louis)
C. Hélène (Paris)
H. Jaenisch (Cambridge, USA)
F. Grosveld (London)
G. Jay (Rockville)
D. Klessig (Piscataway)
P. Leder (Boston)
T. Maciag (Rockville)
D. Miller (Seattle)
R. Mulligan (Cambridge, USA)
S. Rosenberg (Rockville)
K. Singh (Canberra)
A. Stuijte (Amsterdam)
P. Ts'o (Baltimore)
M. Van Montagu (Gent)
E. Wagner (Vienna)
D. Ward (New Haven)
D. Weatherall (Oxford)

The Conference will be held in the Austria Center Vienna and will start around lunchtime on May 18th an finish the evening of May 20th.

There will be a registration fee of US $ 50,–
A number of stipends for substistence or travel of (maximally) öS 3.000,– will be available on application, with first preference given to PhD-students.
Applications for stipends must be accompanied by a supervisor's letter.
Information regarding hotels will be sent when you have registered.

Registration closes March 31st 1990

There ist no application form. Just write to:
Prof. Max L. Birnstiel, I.M.P.,
Dr. Bohr-Gasse 7,
1030 Vienna, Austria
Fax (222) 789390