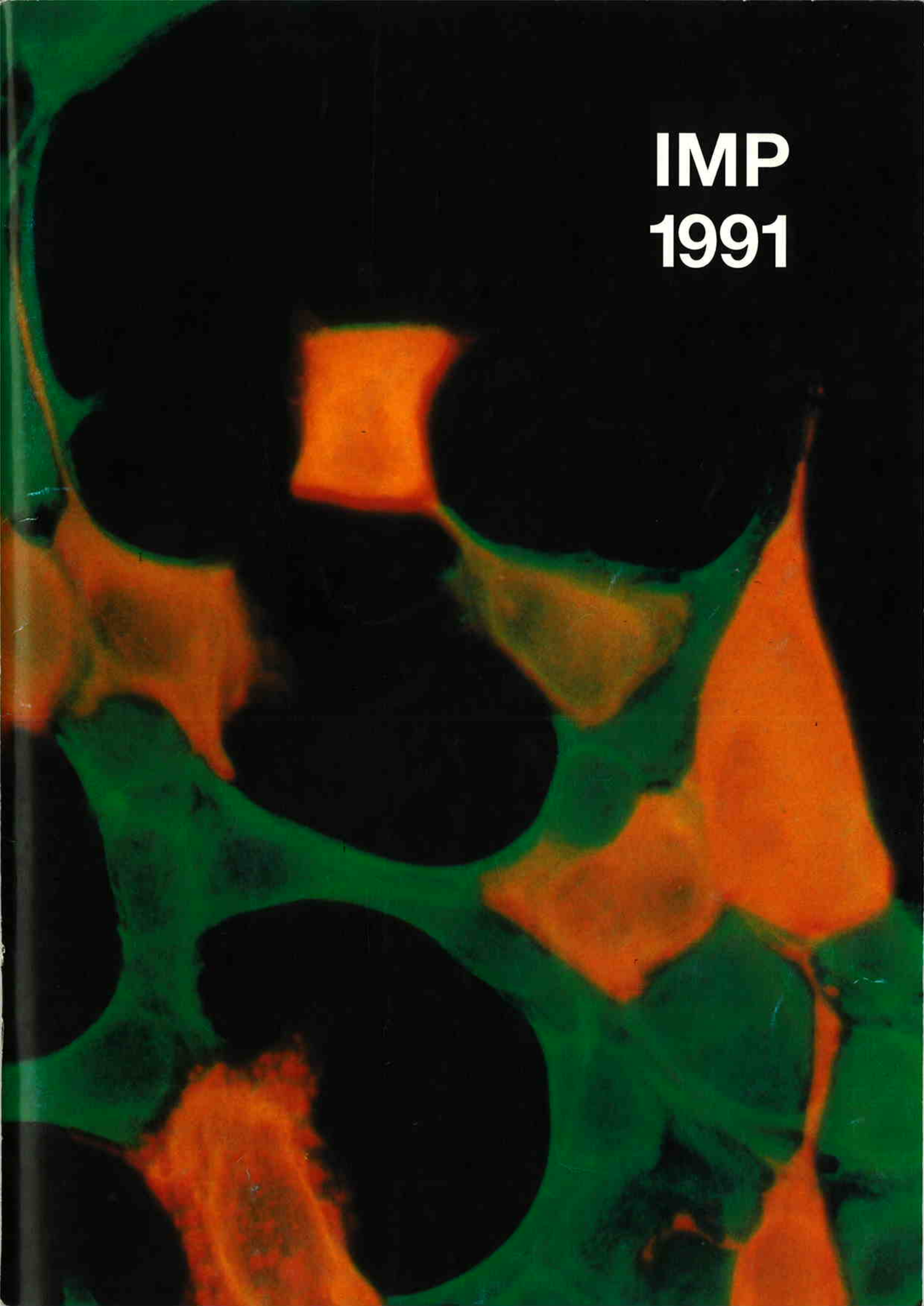
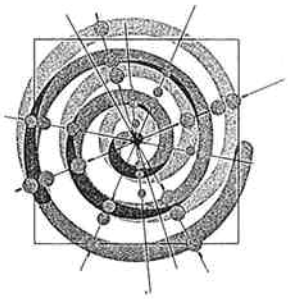

IMP
1991

Scientific Report 1991



Research Institute of Molecular Pathology (I.M.P.)
Dr.-Bohr-Gasse 7
A-1030 Vienna
Austria
Tel. (222) 797 30
Fax (222) 798 71 53

Contents

Impressum

Edited by:	I.M.P. Dr.-Bohr-Gasse 7, A-1030 Vienna, phone: (0043/1) 797 30
Managing Director:	Prof. Dr. Max L. Birnstiel
Administrative Director:	Dr. Nikolaus Zacherl
Coordination and edition:	Dr. Martina Steinhardt
Printed by:	Agens-Werk Geyer + Reiser, 1050 Vienna
Cover:	Transferrinfected mouse hepatocytes
Orange:	human keratin 18
Green:	endogenous mouse keratin
Dye:	indirect double immunofluorescence
Cover photo:	Dr. Kurt Zatloukal, I.M.P.

Research Reports 1991

Max L. BIRNSTIEL	Managing director
Matt COTTEN	Staff scientist
Stephen PHILLIPS	Postdoc
Walter SCHMIDT	Postdoc
Kurt ZATLOUKAL	Postdoc
Harald KANDOLF	PhD-student
Birgit MANDL	PhD-student
Karim TABITI	PhD-student
Helen KIRLAPPOS	Laboratory technician
Karin KOS	Laboratory technician

The work in our group is directed

1. towards development of systems for the delivery of nucleic acids into eukaryotic cells and

2. towards understanding histone sequence diversity during the early sea urchin and Xenopus development.

Novel methods for gene therapeutic intervention

The use of antisense RNA produced by transcription off genes is a principle which has been shown to lead to suppression of unwanted gene activities in transgenic plants and animals. Here the transgene is introduced into the germ line of the organism. The usefulness of this technique for gene therapeutic intervention in somatic cells will depend on whether or not it will be possible to transfer antisense RNA producing genes into chosen tissues. Here, the logistic problems are the same as for experiments in which a gain of function, i.e. the introduction of new gene is required. The method we have developed for this task jointly with Ernst Wagner's group is the receptor mediated introduction of cloned genes into predetermined tissues. Using a variety of receptor ligands in combination with fusogenic principles allowing the transfer of DNA from the endosome to the cytoplasm we have worked out methods for the efficient introduction of cloned DNA into tissue culture cells.

Developmental issues

The second set of projects revolves around developmental questions. Two projects concern the possible function of widely divergent histone proteins. Multicellular organisms often express histone variants in early development which are widely divergent from those of later developmental stages. Histones are usually highly conserved and yet these early histone variants differ by up to 50% and more in their amino acid sequence from histones synthesized late in development. The early histones are present at developmental stages during which new gene expression patterns are established and this raises the question as to whether these histones make a specific contribution to gene control. As first step and as a collaborative venture with M. Busslinger, the chemistry and timing of expression of these early histone proteins are being established in sea urchins. A similar project in collaboration with Drs. Dworkin of the Bender lab concerns H1 variants of *Xenopus* eggs and early embryos. Later we shall make attempts, using antisense methoxy RNA injected into the fertilized *Xenopus* egg, to investigate their role in development. Another ongoing theme is the characterization of the histone 3' processing reaction. Here the U7 snRNP, catalytic unit in this reaction, has been isolated and purified allowing the first biochemical analysis of this small nuclear RNP. Finally, we have investigated whether U7 RNA genes (and other U RNA genes) are amplified during oocyte development. The data show that despite rapid accumulation of the U RNAs in early oocyte development, no gene amplification could be detected for any of the U RNA genes.

Receptor-mediated gene delivery and its enhancement by the endosome-disruption activity of defective adenovirus particles

Matt Cotten, Ernst Wagner, Kurt Zatloukal, Christian Plank, Stephan Phillips, Helen Kirlappos, Karin Kos, David Curiel* and Max L. Birnstiel

* Department of Medicine, The University of North Carolina at Chapel Hill

Receptor-mediated gene delivery

We are developing new methods of introducing DNA into mammalian cells with the goal of generating a safe and reliable method that introduces DNA into a high percentage of the target cells. Our strategy has been to pirate the receptor-mediated endocytosis route to deliver our DNA. This approach uses various polycation-ligand chemical conjugates, such as transferrin-polylysine (1–5), which serve to condense the transferred DNA into compact, ligand-coated donuts, comparable in size to a typical DNA virus (6, see figure 1).

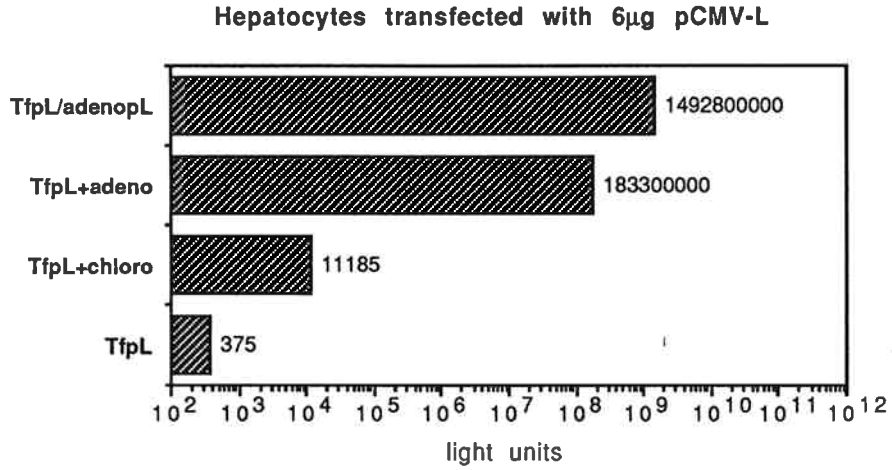


Figure 1. *Impact of chloroquine, free adenovirus and enzymatically polylysine-modified adenovirus on expression of a luciferase reporter gene construct (light units). $3x10^5$ mouse hepatocytes were transfected with 6 µg pCMV-L complexed to transferrin-polylysine conjugates (Tfpl). Alternatively, transfection was performed in the presence of 100 µM chloroquine (Tfpl+chloro), in the presence of free replication defective adenovirus (Tfpl+adeno) or with polylysine-modified adenovirus, which had been directly attached to DNA-transferrin-polylysine conjugates (Tfpl/adenopl).*

We have demonstrated both transient and stable gene delivery into a variety of cell types. In certain cell lines, such as K562 cells, we can demonstrate transient gene expression in greater than 90% of the target cells (5). Stable expression of introduced genes can also be demonstrated, with 0.5–1% of the initial transfected cell population recoverable as stable expressing clones.

The Ligands

Our initial experiments concentrated on transferrin-polylysine and conalbumin-polylysine conjugates to delivery DNA via the transferrin receptor, which is abundant on most proliferating cells. Additional experiments have shown that either natural or synthetic ligands which bind to the hepatocyte-specific asialoglycoprotein receptor can replace transferrin and can be used to target genes to these cells (see Kurt Zatloukal's report and the report from the Ernst Wagner group for details). Using antibodies which recognize various T-cell surface proteins (CD4, CD7) and a viral glycoprotein which binds the CD4 molecule (the HIV gp120 molecule) polylysine conjugates which deliver DNA via the CD4 or CD7 molecules have also been tested.

Adenovirus enhancement of receptor-mediated gene delivery

One of the major limits to gene delivery by endocytosis in many cell types may be the exit of the endocytosed material from the endosome. However, many viruses are known to enter cells via receptor-mediated endocytosis and possess molecular skills to mediate disruption of the endosomes. We wondered if the endosome disruption activity of a virus or a viral protein might function in trans to facilitate the co-entry of receptor-bound DNA particles.

To test this idea, plasmid DNA containing a marker gene is complexed and condensed into a compact donut with polylysine covalently linked to the cell binding ligand such as transferrin. When these ligand-coated DNA donuts are supplied to the appropriate cells in the presence of a replication-defective adenovirus, both the DNA donut and the virus are endocytosed, and the low pH-triggered adenoviral endosome disruption allows the efficient cellular entry of the the DNA donut (see figure 1). We find that this maneuver enhances delivered gene expression 100–1000 fold in a variety of cell types (7, 8). Furthermore, a large percentage (70–100%) of the target cells (HeLa cells or hepatocytes, see Kurt Zatloukal section below) can be found to transiently express the marker gene.

Modifications of the virus-in-*trans* idea

This use of a virus in *trans* solves many of the safety and practical problems associated with using recombinant viruses to deliver genes. In this context, the virus is functioning merely as an endosomolytic agent. Therefore we can use a replication defective virus and we can treat the virus with various agents which further block replication possibilities, such as UV irradiation, formaldehyde or psoralen+UV. As long the virus particle maintains its endosome disruption activity it is useful in this application (ref. 10). Eventually we hope to use the purified endosome disrupting activity from the adenovirus particle.

Because the virus particle is functioning in *trans*, this system allows great size and sequence variety in the DNA to be delivered; in contrast to the 6–8.5 kilobase size limit of the standard recombinant adenovirus vectors, we routinely deliver 15 kb plasmids and we have demonstrated functional delivery of 48 kb DNA molecules. The high efficiency delivery of a 48 kb, luciferase-encoding cosmids suggests that very large DNA molecules can be delivered with this method if special care is given to the preparation and condensation of the large DNA (see the section of Stephen Phillips and ref. 10). Therefore, we are now testing the delivery of a 150 kb yeast artificial chromosome (YAC).

Coupled adenovirus in *cis*

Our initial application of the endosome disrupting virus required two receptor-binding and endocytosis events: the adenovirus must bind to its receptor as well as the transferrin-DNA complex must bind to its receptor (see Fig. 3). Directly coupling the virus to the condensed DNA complex results in a further improvement

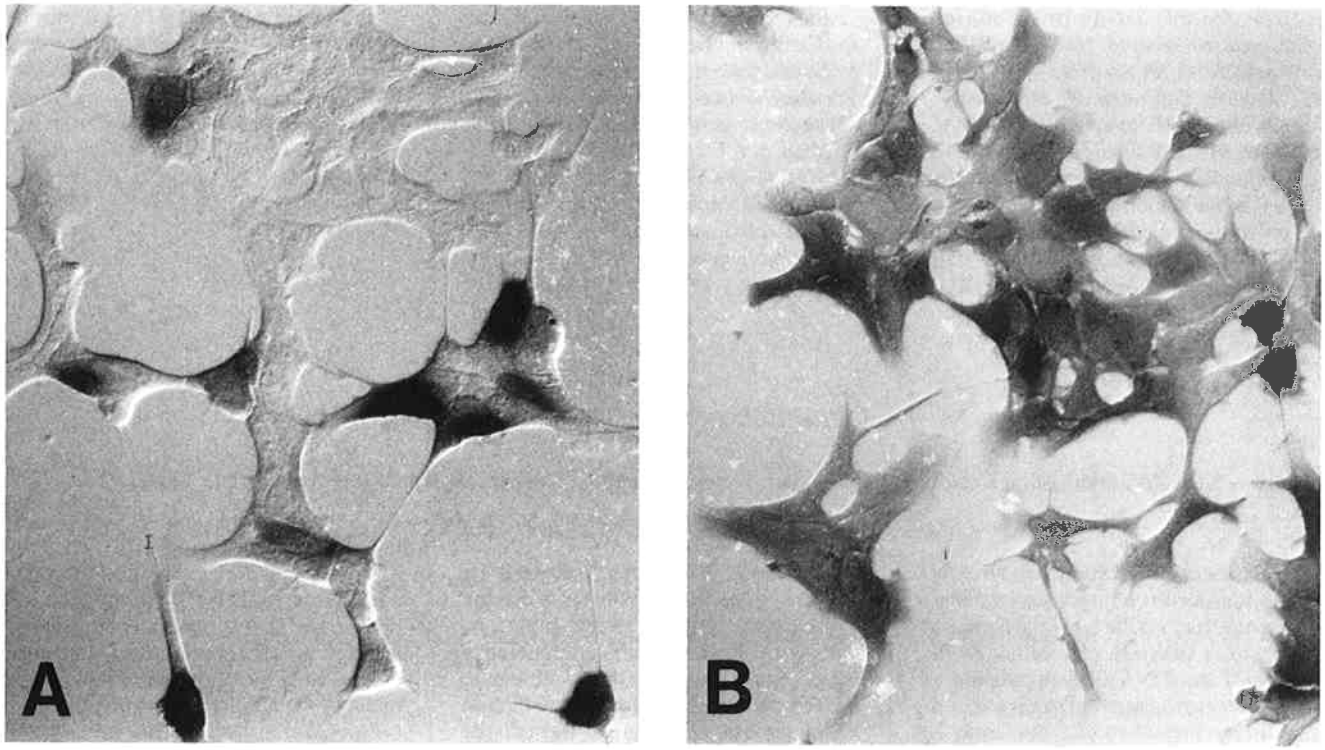


Figure 2. *Histochemical detection of the expression of a β-galactosidase reporter gene in mouse hepatocytes. The cells had been transfected with 6 μg pCMV-β-gal complexed to transferrin-polylysine and addition of free adenovirus (A) or complexed to transferrin-polylysine and polylysine-modified adenovirus (B). Note, that in the transfection performed with the polylysine-modified adenovirus almost all hepatocytes were positively stained for β-galactosidase. A and B x 174.*

in the DNA delivery. The coupling can be accomplished by several different methods including using a polylysinated-antibody bridge (8), by direct coupling of polylysine to the adenovirus using either enzymatic (see Kurt Zatloukal's section) or chemical crosslinking, or by biotinylating the adenovirus particle and using a streptavidin-polylysine conjugate (see the report of Ernst Wagner and ref. 9). The transported DNA is then complexed with the polylysine virus. Additional polylysine-ligands such as transferrin-polylysine can then be added to the complex. This direct coupling of the virus to the transported DNA has a number of advantages over the use of free adenovirus. The quantity of DNA and virus required to transform a population of cells is drastically reduced. Because the virus and the DNA are physically bound, their co-entry into the same endosome is ensured and high level gene expression can be demonstrated with as little as 1 virus and 10 DNA molecules per cell. This may be especially important for *in vivo* applications. Combination complexes can be prepared with DNA donuts containing a cell-targeting ligand (such as transferrin, an antibody or an artificial ligand for the asialoglycoprotein receptor) plus polylysine-adenovirus. These complexes can now bind and enter cells that express little or no adenovirus receptor using the new cell binding ligand and the adenovirus particle functions only as an endosome disruption agent.

Conclusions and Further Directions

Our current efforts are directed at the following problems: 1. How suitable is the adenovirus system for generating stable clones? Do our inactivation protocols sufficiently block adenovirus replication to ensure the absence of adenovirus in the resulting clones? 2. Can this system be used *in vivo* by either direct application to the blood stream, infusion into the lung, or topical application? 3. Is the system functional with explants of primary cells such as bone marrow or primary hepatocytes? Initial experiments have demonstrated transient expression with both mouse embryonic stem cells and with mouse bone marrow cells. These results would support an *ex vivo* application of the system.

This use of a defective virus particle in *trans* represents a new class of gene delivery techniques. It combines the entry efficiency of a virus with the flexibility and safety of a number of non-viral methods. Although there are still a number of problems to be solved with this method, we believe that it provides a new way of thinking about DNA delivery and facilitates a variety of possibilities for gene therapy applications.

REFERENCES

1. *Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L. (1990): Transferrin-polycation conjugates as carriers for DNA uptake into cells. Proc. Natl. Acad. Sci. USA 87, 3410–3414.*
2. *Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M. L. (1990): Receptor-mediated endo-cytosis of transferrin polycation conjugates: An efficient way to intro-*

duce DNA into hematopoietic cells. Proc. Natl. Acad. Sci. USA **87**, 3655–3659.

3. Cotten, M., Langle-Rouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., and Birnstiel, M. L. (1990): Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. Proc. Natl. Acad. Sci. USA **87**, 4033–4037.
4. Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991b): DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. Bioconjugate Chemistry **2**, 226–231.
5. Cotten, M., Wagner, E., and Birnstiel, M. L. (1991): Receptor-mediated transport of DNA into eukaryotic cells. Methods Enzymol. in press.
6. Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991): Transferrin-poly-cation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. Proc. Natl. Acad. Sci. USA **88**, 4255–4259.
7. Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991): Adenovirus enhancement of transferrin-polylysine mediated gene delivery. Proc. Natl. Acad. Sci. USA **88**, 8850–8854.
8. Curiel, D., Wagner, E., Cotten, M., Birnstiel, M. L., Li, C., Loechel, S., Agarwal, S., and Hu, P. (1991): High efficiency gene transfer mediated by adenovirus coupled to DNA polylysine complexes via an antibody bridge. J. Biol. Chem. submitted.
9. Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D., and Birnstiel, M. L. (1991): Coupling of adenovirus and polylysine-DNA complexes greatly enhances receptor-mediated endocytosis and expression of transfected genes (in preparation).
10. Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D., and Birnstiel, M. L. (1991): The use of defective and chemically-inactivated adenovirus particles in trans to trigger endosomolysis and augment receptor-mediated gene delivery (in preparation).

Gene delivery to hepatocytes

Kurt Zatloukal, Matt Cotten, Ernst Wagner, Helen Kirlappos, Karin Kos, Karl Mechtler, Max L. Birnstiel

Gene-constructs complexed to polylysine-transferrin conjugates are taken up by hepatocytes via the receptor-mediated endocytosis pathway (1). Although the uptake of the DNA in a population of cultured hepatocytes is rather even, as shown by histochemical detection of biotinylated DNA, only very few of them (less than one in a thousand) express the delivered gene. One possibility is that the high-capacity lysosomal degradation system of hepatocytes is the major hindrance to successful gene transfer. A dramatic increase in the expression of a delivered luciferase reporter gene is achieved (see Fig. 1) when the transfection is performed in the presence of replication-defective adenovirus (dl312) (2). Histochemical detection of the expression of a β-galactosidase reporter gene revealed, however, that even in the presence of adenovirus only 5%–10% of the hepatocytes were positively stained (Fig. 2a). An explanation for this restricted expression is that

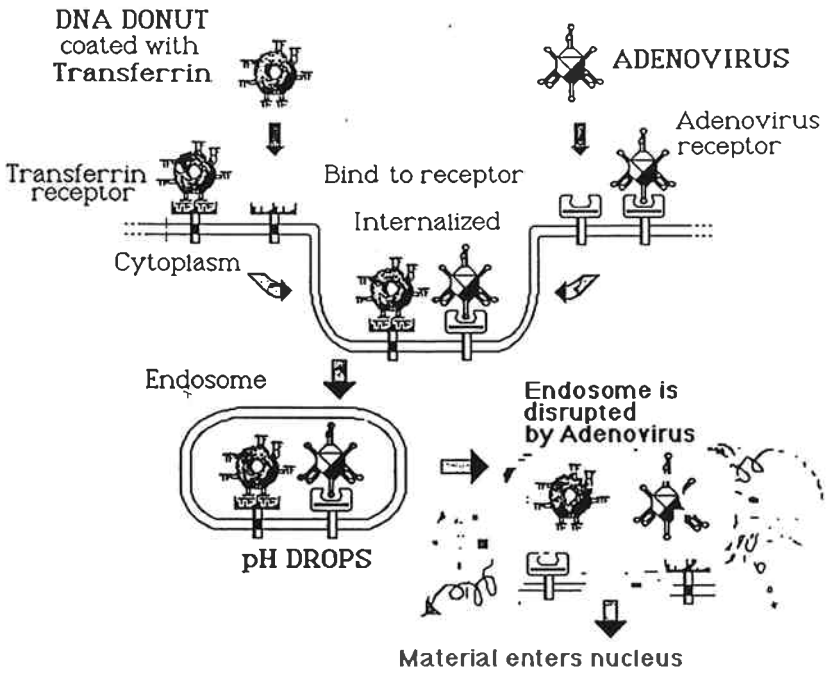


Figure 3. *Cartoon of steps in transferrinfection using linked virus*
DNA is combined with virus-polylysine conjugate which neutralizes part of the charges on the DNA. Transferrin-polylysine is then added to neutralize the remainder of the charges. The ternary complex consisting of virion-polylysine, transferrin-polylysine and DNA attaches to the transferrin receptor, gets internalized into endosomes where the endosomolytic function of the virion releases the DNA complexes into the cytoplasm.

hepatocytes are rather poor targets for adenovirus infection and that adenovirus can only exert its effect if it is cointernalized with the condensed gene-construct. This is further supported by the observation that HeLa cells, which are highly susceptible to adenovirus, can be transfected under these conditions with almost 100% efficiency.

To optimize the system for cells which have few or no receptors for adenovirus, we directly attached the adenovirus to the gene-constructs. This was achieved by covalently coupling polylysine to glutamine residues of the viral capsid proteins with the help of transglutaminase. For transfection of hepatocytes, DNA-complexes consisting of polylysine-modified adenovirus and polylysine-modified transferrin were used; the whole virus-containing complex can now be taken up by the transferrin receptor and/or adenovirus receptor. With these complexes more than 90% of the treated hepatocytes express the delivered gene-construct at a very high level (Figs. 1 and 2b). Moreover, the transfection efficiency was sufficiently high that useful gene expression was obtained even when a very low amount of DNA had been applied (see report from the group Ernst Wagner for more details).

On one hand, this new gene transfer system combines the high efficiency of viral vectors and on the other hand the advantages of receptor-mediated gene delivery, like gene transfer to non-dividing cells, no restriction for the size of the DNA (up to 50 kb), suitable for cotransfer of different gene-constructs and ready expression from different promoters. We are currently testing the system in various experimental applications and for its applicability for gene therapy.

REFERENCES

1. *Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991): Bioconjugate Chem* **2**, *226–231.*
2. *Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991): Proc. Natl. Acad. Sci. USA* **88**, *8850–8854.*

Delivery of Large DNA Molecules to Eukaryotic Cells by Adenovirus Aided Transferrinfection

Stephen Phillips, Matt Cotten, Kurt Zatloukal, Ernst Wagner and Max L. Birnstiel

The receptor-mediated gene delivery system termed *transferrinfection* (1) has been shown to be an effective method for introducing DNA molecules into a variety of eukaryotic cell lines, especially those derived from the erythroid lineage (1–3). A number of other cell lines have, however, proved to be either partially or wholly resistant to transfection by this method (5). The recent introduction of transferrinfection in the presence of a replication-defective adenovirus (7), which functions as an endosome disrupting agent, now enables those cell lines for which transferrinfection alone was of limited or no use to be efficiently transfected.

A further limitation of the original transferrinfection method was its incapacity to efficiently deliver large DNA molecules (>15 kbp) to cells (5). Since several genetic abnormalities are associated with genes encoded by large DNA segments there is an obvious need in gene therapy for a delivery system capable of efficiently introducing large DNA molecules into cells. We have now demonstrated that adenovirus aided transferrinfection, with slight modification of the DNA condensation conditions, allows for the efficient and functional delivery of DNA molecules up to 48 kbp to a number of different cell lines.

A reporter gene (*P. pyralis* luciferase) was inserted into a cosmid clone, giving a total size of 48 kbp, and the efficiency of DNA delivery by transferrinfection and adenovirus aided transferrinfection compared directly with that of a plasmid construct (12 kbp) containing the same reporter gene. In the cell lines so far tested (e.g. HeLa cells, mouse fibroblasts and a human neuroblastoma cell line) the delivery of cosmid DNA molecules, as determined by luciferase activity, could be enhanced by greater than 103 to 104 fold with the virus in *trans* (essentially zero without adenovirus) and a further 10 fold maximally with the virus in *cis*. On a molar basis the efficiency of gene delivery for the cosmid, as compared to the plasmid, was the same or only diminished by a maximum of 2 fold. Therefore, since we know that a large proportion of the test cells can be efficiently transfected with plasmid DNA constructs, e.g. HeLa cells (see Kurt Zatloukal's report), it appears as though the cosmid DNA is also being efficiently delivered to a large proportion of the cells, however, this remains to be tested directly.

We are currently investigating the potential of this new approach to deliver a 150 kbp yeast artificial chromosome (YAC) and its suitability for generating cells stably transformed with a gene encoded by a large DNA molecule.

For references see contribution of M. Cotten *et al.*, page 6

The bcr-abl fusion sequence as target for inhibition by antisense DNA and RNA

Walter Schmidt and Max L. Birnstiel

The bcr-*abl* gene product is a fusion protein which can be detected in almost every patient with chronic myelocytic leukemia (CML) and is strongly related to this disease (1). Its inhibition might be helpful in therapy of CML patients.

The bcr-*abl* protein is expressed after a chromosomal translocation resulting in the production of a chimaeric bcr-*abl* mRNA with a specific bcr-*abl* joining sequence. The aim of this project is to realize a way for inhibition of bcr-*abl* gene product by antisense DNA, RNA, catalytic ribozymes and chemically modified variants of them.

In order to identify the most efficient approach for inhibition a model system has been established. As target for antisense inhibition a vector which carries the bcr-*abl* joining sequence upstream of the coding region of the luciferase gene has been stably introduced into the CML cell line K562 (K562ba-L). After incubation with bcr-*abl* antisense oligonucleotide reduction of luciferase activity has been observed, indicating an inhibitory effect on the bcr-*abl* part of the luciferase mRNA. Another possible approach is the use of antisense RNA. A vector expressing the bcr-*abl* antisense RNA under the control of the HCMV promoter was transiently transfected into K562ba-L cells. The transferrin receptor mediated gene transfer system allowed delivery of the construct to a high percentage of the cell population (2). Luciferase activity was transiently reduced whereas transfection of the control plasmid lacking the bcr-*abl* sequence did not show any effect. Therefore, it might be possible that constitutively expressed bcr-*abl* antisense RNA stably inhibit bcr-abl fusion sequences.

REFERENCES

1. *DeKlein, A., van Kessel, A. G., Grosveld, G., Bartram, C. R., Hagemeijer, A., Bootsma, D., Spurr, N. K., Heisterkamp, N., Groffen, J., and Stephenson, J. R. (1982): Nature* ***300****, 765-767.*
2. *Cotten, M., Laengle-Rouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., and Birnstiel, M. L. (1990) Proc. Natl. Acad. Sci. USA 87, 4033–4037.*

Histone H1 in early embryogenesis of Xenopus laevis

Harald Kandolf, Eva Dworkin-Rastl* and Max Birnstiel

* Ernst Boehringer Institute, Vienna

In addition to its function as a general repressor of gene expression by forming highly condensed chromatin, a possible role for histone H1 in the control of individual genes has to be considered. Moreover, the functional significance of H1 polymorphism is still an open question. Three H1 variants are reported to be present in all tissues of *Xenopus laevis* (H1A, H1B and H1C), H1A always being the predominant subtype. In the pre-gastrula embryo H1 is clearly under-represented compared to the nucleosomal histones making the time point of the mid blastula transition (MBT) most interesting, after which differentiation starts and a boost in H1 synthesis is observed. There is much controversy in the literature about H1 quantity and localization in eggs and cleavage-stage (CS) embryos. Hence an accurate analysis is necessary prior to further experiments which will comprise of repression and overexpression of single H1 variants in this system.

We have raised polyclonal antibodies against H1A, H1B, H1C and the proposed maternal H1 variant B4 by using small terminal peptides for rabbit immunization. To look for H1 localization the resulting sera were used for an *in situ* immunofluorescence analysis (FITC) of *Xenopus* embryo paraffin sections. We observed a very significant staining of chromosomes with the B4 antiserum with CS embryos, remaining roughly constant up to early gastrula. H1A was not detectable at CS, rather faint but significant at blastula and bright at gastrula, reflecting the boost in synthesis. The H1B and H1C antisera did not stain any of the stages investigated.

In order to get accurate data of protein amounts in these early stages we developed a quantitative immunoblot assay. Expression vectors containing the published H1 genes were constructed. As a reference for quantification, radioactively labelled proteins synthesized *in vitro* in reticulocyte lysate were used. The affinity purified H1A antibody detects as little as 100 pg protein in immunoblot experiments using 125J-labelled protein A.

A significant amount of H1A could not be detected in pre-MBT embryos which suggests that the actual maternal store of H1A is much smaller than the reported 8-10 ng per unfertilized egg (van Dongen et al., 1983). The B4 protein, which is highly homologous to the sea urchin CS H1 protein, has a maternal store of 1-2 ng (R. Smith, unpublished result).

These findings support the idea that B4 could functionally replace H1 in the CS embryo. In spite of the high specific activity of H1B and H1C antibodies these variants could not be detected in significant amounts (<1 pg) even in neurula embryos.

REFERENCES

van Dongen, W. M., Moorman, A. F., and Destree, O. H.: "The accumulation of the maternal pool of histone H1A during oogenesis in Xenopus laevis". Cell Differentiation ***12*** *(1983) 257–264.*

cDNA-cloning of oocyte-specific histones of the sea urchin

Birgit Mandl, Kurt Zatloukal, Wolfgang Brandt* and Meinrad Busslinger
*University of Cape Town, South Africa

The so-called cleavage stage (CS) histones are the predominant histones in the chromatin of the egg and the early cleavage-stage embryo of the sea urchin. They are synthesized during oogenesis resulting in a large maternal store in the egg and during the first few cell divisions in the cleaving embryo where they were first described.

CS histones are not only responsible for the condensation of the maternal chromosomes in the oocyte, but after fertilization the maternally stored CS histones replace the sperm specific histones in the male pronucleus leading to extensive chromatin remodelling and subsequent trans-criptional activation of the paternal ge-nome (1).

The CS histone genes resisted all conventional cloning attempts based on screening with heterologous histone gene probes suggesting that they differ considerably from other sea urchin histone variants.

We have biochemically purified the CS histones to homogeneity and partial protein sequence data obtained by microsequencing were used to design specific PCR-primers that allowed PCR-cloning of all five CS histone cDNAs from *P. miliaris* egg poly(A)+ RNA.

Presently we are analyzing the cDNA clones with particular attention to CS H1 because of the function of H1 histone as important factor in gene regulation.

The 3 kb long CS H1 mRNA contains exceptionally long leader and trailer sequences and encodes the longest H1 protein known. The mRNA is detectable only in the egg and in the embryo up to early blastula stage. Sequence comparisons reveal low homology (about 40%) between the CS H1 protein and all known H1 variants of the sea urchin. Interestingly, a 60% homology is observed with the *Xenopus laevis* B4 protein (Smith et al., 1988; see report Harald Kandolf). B4 is the early embryonic H1 histone in *X. laevis*. B4 is almost as long as CS H1 and shows an expression pattern similar to that of *P. miliaris* CS H1. Additionally the B4 and CS H1 genes both code for polyadenylated mRNAs and therefore belong to the class of replacement histone genes, whose expression is indepented of U7-processing and the cell cycle.

Considering these facts we suggest that the B4 protein may be the vertebrate homologue of the CS H1 histone.

REFERENCES

1. *Poccia, D. (1986): Remodeling of nucleoproteins during gametogenesis, fertilization and early development. Int. Rev. Cytol.* ***105****, 1–65.*
2. *Smith, R. C., Dworkin-Rastl, E., and Dworkin, M. B. (1988): Expression of a histone H1-like protein is restricted to early Xenopus development. Genes Dev.* ***2****, 1284–1295.*

Amphibian Oocytes, Sphere Organelles and U snRNA Genes

Stephen Phillips, Matt Cotten, Françoise Laengle-Rouault, Gotthold Schaffner and Max. L. Birnstiel

The 28S, 18S and 5.8S rRNA genes of *Xenopus* are amplified approximately 1000 fold during oogenesis, this being the classical example of gene amplification (1). The extrachromosomal rDNA copies generated as a result of this amplification are situated and transcribed in the numerous nucleoli found in the nucleoplasm of the mature oocyte. In view of the parallels between the amphibian oocyte nucleoli and sphere organelles, the latter recently shown to contain small nuclear ribonucleoprotein particles (snRNPs) (2), and the short time span allowed for the synthesis of the relatively large amounts of U snRNAs accumulated prior to vitellogenesis, we decided to investigate whether or not the U snRNA genes are amplified during *Xenopus* oogenesis. The sphere organelles being possible sites for the location and transcription of such extrachromosomal U snDNA copies.

To this end we carried out a series of quantitative nucleic acid hybridization experiments designed to accurately measure the degree of gene amplification and determine the methylation status of the U snDNAs in *Xenopus* oocytes relative to somatic (blood) cells. These included titration of the U snRNA genes in DNA from isolated oocyte nuclei (germinal vesicles) against DNA from somatic cells, quantitative Southern blotting and analyses of buoyant density gradient fractions of oocyte and somatic DNA samples. The hybridization probes we used were 32P labelled *X. laevis* U snDNAs (U1, U2, U4, U5, U6 and U7) and the 18S and oocyte-type 5S rDNAs, the latter serving as internal amplification and non-amplification controls respectively against which all other hybridization signals were standardized. Heterologous human U3 and U11 and rat U8 snDNA probes were also employed but yielded no significant hybridization signals, this suggesting that the *Xenopus* analogues are somewhat divergent. In brief, the combined results from these studies demonstrate that the U snRNA genes investigated are not amplified in *Xenopus* oocytes, therefore, the sphere organelles cannot contain extrachromosomal U snDNA copies (3). However, our findings do not rule out the possibility that the sphere organelles might contain amplified copies of the, as yet uncharacterized, sphere organizer DNA. During the above studies we also isolated and partially characterized 9 different U7 snRNA gene containing clones from *X. laevis*. The U7 snRNA being a key component of the RNA processing machinery involved in generating the mature 3' ends of the replication-dependent histone mRNAs (4). The sequences so far obtained reveal that the U7 snRNA genes, ~50 per haploid genome, are clustered but not tandemly reiterated as is the case with the major U snRNA genes. Further sequence analysis indicates that the promoter structure of this minor U snRNA gene is similar to the vertebrate major U snRNA genes, i.e. a downstream 3' box (+7 to +19) and an upstream proximal sequence element (PSE) (–52 to –62). However, the distal sequence element (DSE) of the *Xenopus* U7 snRNA gene is composed of two inverted octamer-binding motifs separated by 3 nucleotides and located, unusually, 4 nucleotides upstream of the PSE. We have demonstrated that the *X. laevis* oocyte contains a corresponding RNA species that is 57 nucleotides long, the synthesis of which plateaux at ~4 x 10^6 transcripts/oocyte between stages II and III during oogenesis.

REFERENCES

1. *Bird, A. P. (1980):Gene Reiteration and Gene Amplification. Cell. Biol.* ***3****, 362–111.*
2. *Gall, J. G. (1991): Spliceosomes and Snurposomes. Science* ***252****, 1499–1500.*
3. *Phillips, S. C., Cotten, M., Laengle-Rouault, F., Schaffner, G., and Birnstiel, M. L. (1991): Amphibian Oocytes and Sphere Organelles: Are the U snRNA Genes Amplified? Chromosoma, submitted.*
4. *Birnstiel, M., L., and Schaufele, F. (1988): in Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Birnstiel, M. L. (ed.), (Springer, Heidelberg) pp. 155–182.*

Biochemistry of the Histone RNA 3' Formation

Karim Tabiti, Hamilton O. Smith and Max L. Birnstiel

Levels of histone mRNAs are regulated during the cell cycle, apparently as a consequence of transcriptional and especially posttransscriptional regulation (1). In contrast to most other transcripts maturation of histone mRNA does not require splicing or polyadenylation reactions. Histone mRNA processing consists solely of an endonucleic cleavage between two highly conserved sequence elements.

Recent results have demonstrated that multiple factors are involved in the formation of translatable species of histone mRNA. These include a hairpin-binding factor (2), a heat-labile factor whose steady state levels are regulated during the cell cycle (3) and a low-abundance small ribonucleoprotein particle (snRNP) of the Sm class called U7 (4).

A basic cleavage pathway has been defined, in which the RNA moiety of the U7 snRNP forms an RNA-RNA duplex with the conserved processing signal a few nucleotides downstream the actual cleavage site. Concomitantly the conserved upstream processing signal is bound by the hairpin binding factor which presumably stabilizes the interaction between histone precursor and U7 snRNP.

In contrast to splicing reactions and 3'end formation of polyadenylated mRNA, *in vitro* processing of primary histone transcripts may be carried out in the presence of metal chelating agents and in the absence of nucleotide triphosphates, indicating that a unique recognition, cleavage and regulatory mechanism is required for the 3' processing of histone mRNA.

We are interested in detecting factors involved in the processing reaction and assigning catalytic or regulatory functions to them with the ultimate aim of understanding on a molecular level the mechanism of 3' processing of histone mRNA.

Identification and Purification of Processing Components

In intitial attempts to identify processing components, we fractionated EBI mouse cell nuclear extracts in a large scale over various FPLC columns. *In vitro* pro-cessing reactions and complementation assays allowed us to monitor activity of the heat-labile factor, the hairpin binding factor and the U7 snRNP during purification. Purification of these factors by conventional methods proved to be a formidable undertaking because of their low abundance. Nevertheless, this approach resulted in a partial purification of the heat-labile factor which was free of detectable amounts of U7 snRNA. The processing components comigrated over a few thousand fold purification, suggesting that most of the processing may be tightly bound to the U7 snRNP or are integral parts of the particle, but that a small fraction of the associated factors may be stripped of during some purification steps.

We have now developed a two-step affinity purification which allows us to specifically isolate and characterize minor snRNPs (5). This procedure involves annealing and melting of the U7 snRNP to 2'O-methyl ribonucleotide decamers. Analysis of the purified fractions reveals that the U7 snRNP contains a full complement of standard core proteins in addition to two U7-specific polypeptides with apparent molecular weights of 14 kDa and 50 kDa. Further we have established an assay which allows us to monitor the quantity, integrity and molecular weight of the particles during manipulation.

Structural analyses of the U7 snRNP using base-specific chemical probes corroborate former studies which indicate that the RNA is extensively complexed with proteins but accessible for base pairing at the 5'end (6). Despite the fact that our study failed to detect any changes at the 5' end during the switch from exponentially growing to quiescent cells, there are some lines of evidence that indicate that the 5' end of U7 snRNA may be involved in the cell cycle dependent expression of histone proteins (7).

In future experiments we will focus our attention to the interaction between hairpin-binding factor and the upstream conserved sequence element of the histone primary transcript. Gel retardation assays performed by us and others (8) have revealed a 50 kDa protein, which specifically binds to the upstream conserved sequence element. Several lines of evidence suggest that this may be one of the identified U7-specific proteins and that there is a tight functional relationship between this 50 kDa protein and the heat labile factor which participates in the overall regulation of histone mRNA processing.

REFERENCES

1. *Schümperli, D. (1988): Multilevel regulation of replication dependent histone genes. Trends Genet.* ***4****, 187–191.*
2. *Vasserot, A. P., Schaufele, F. J., and Birnstiel, M. L. (1989): Conserved terminal hairpin sequences of histone mRNA precursors are not involved in duplex formation with U7 RNA but act as a target site for a distinct processing factor. Proc. Natl. Acad. Sci. USA* ***86****, 4345–4349.*
3. *Gick, O., Krämer, A., Vasserot, A. P., and Birnstiel, M. L. (1987): A heat labile regulatory factor is required for 3' processing of histone pre-mRNAs. Proc. Natl. Acad. Sci. USA* ***84****, 8937–8940.*
4. *Birnstiel, M. L., and Schaufele, F. J. (1988): Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles, ed. Birnstiel, M. L., 155–182.*
5. *Smith, H. O., Tabiti, K., Schaffner G., and Birnstiel, M. L. (1991): Two step affinity purification of U7 small nuclear ribonucleoprotein particles using complementary biotinylated 2'-O-methyl oligoribonucleotides. Proc. Natl. Acad. Sci. USA., in press.*
6. *Tabiti, K., Smith, H. O., Schaffner., G., and Birnstiel, M. L. Structural analyses of U7 small nuclear ribonucleo-protein particle (snRNP) using base specific chemical probes. In preparation.*
7. *Hoffman, I., and Birnstiel, M. L. (1990): Cell cycle-dependent regulation of histone precursor mRNA processing by mmodulation of U7 snRNA accessibility. Nature* ***346****, 665–668.*
8. *Pandey, N. B., Sun J., and Marzluff, W. F. (1991): Different complexes are formed on the 3' end of histone mRNA with nuclear and polyribosomal proteins. Nucleic Acids Res.* ***20****, 5653–5659.*

PUBLICATIONS SUBMITTED 1991

1. *Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991): Transferrinpolycation DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc. Natl. Acad. Sci. USA* ***88****, 4255–4259.*
2. *Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991): DNA-Binding Transferrin Conjugates as Functional Gene Delivery Agents: Synthesis by Linkage of Polylysine or Ethidium Homodimer to the Transferrin Carbohydrate Moiety. Bioconj. Chemistry,* ***2****, 226–231.*
3. *Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, Ch., Schaffner, G., Wagner, E., and Birnstiel, M. L. (1991): 2'-0-Methyl, 2'-0-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribonucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA pro-cessing event. Nucl. Acids Res.* ***19****, 2629–2635.*
4. *Cotten, M., Wagner, E., and Birnstiel, M. L. (1991): Receptor Mediated Transport of DNA into eukaryotic cells. A chapter in Methods in Enzymology, March 1991, in press.*
5. *Eckner, R., Ellmeier, W., and Birnstiel, M. L. (1991): Mature mRNA 3' end formation stimulates RNA export from the nucleus. EMBO J.* ***10****, 3513–3522.*
6. *Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991): Adenovirus enhancement of transferrin-polylysine mediated gene delivery. Proc. Natl. Acad. Sci. USA* ***88****, 8850–8854.*
7. *Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M., and Noe, Ch. R. (1991): A simple procedure for the preparation of protected 2'0-methyl or 2'0-ethyl ribonucleoside-3'-0-phosphoramidites. Nucl. Acid Res., in press.*
8. *Smith, O., Tabiti, K., Schaffner, G., and Birnstiel, M. L. (1991): Two-step affinity purification of U7 small nuclear ribonucleoprotein particles using complementary biotinylated 2'0-methyl oligoribonucleotides. Proc. Natl. Acad. Sci. USA, in press.*
9. *Phillips, St., Cotten, M., Laengle-Rouault, F., Schaffner, G., and Birnstiel, M. L. (1991): Amphibian oocytes and sphere organelles: are the U snRNA genes amplified? Submitted to Chromosoma.*
10. *Curiel, D. T., Agarwal, S., Romer, N., Wagner, E., Cotten, M., Birnstiel, M. L., and Boucher, R. C. (1991): Gene transfer to respiratory epithelial cells via the receptor-mediated endocytosis pathway. Submitted to American Review of Respiratory Cell and Molecular Biology.*
11. *Curiel, D. T., Wagner, E., Cotten, M., Birnstiel, M. L., Li, Ch., Loechel, St., Agarwal, S., and Hu, P. (1991): High efficiency gene transfer by adenovirus coupled to DNA-polylysine complexes via an antibody bridge. Human Gene Therapy, in press.*
12. *Zatloukal, K., Wagner, E., Cotten, M., Phillips, St., Plank, Ch., Steinlein, P., Curiel, D., and Birnstiel, M. L. (1991): Transferrinfection: a highly efficient way to express gene constructs in eukaryotic cells. Submitted to the Annals of the New York Academy of Sciences.*
13. *Eckner, R., and Birnstiel, M. L. (1991): Evolutionary conserved multiprotein complexes interact with the 3' untranslated region of histone transcripts. Submitted to Nucl. Acids. Res.*

The Senior Scientists

Conditional oncogenes as tools to study oncogenesis *in vitro*

Hartmut BEUG (October 1988)	Senior scientist
Christian SCHROEDER (until August 1991)	Postdoc
Ernst REICHMANN (March 1989)	Postdoc
Peter STEINLEIN (January 1990)	Postdoc
Irene LEITNER (March 1990)	Ph D-student
Oliver WESSELY (May 1991)	Ph D-student
Alberto MUNOZ (until Sept. 1991)	Guest scientist
Leonie GIBSON (April 1990)	Technician
Evi DEINER (October 1990)	Technician

Introduction:

In 1991, we have continued our work on selected aspects of the AEV system (in collaboration with the group of Martin Zenke). As described in the 1990 report, we try to elucidate mechanisms by which the two oncogenes of the AEV virus, a mutated epidermal growth factor receptor (v-*erb*B) and a mutated nuclear thyroid-hormone receptor (v-*erb*A) convert normal erythroid progenitors into leukemic cells. In 1991, we have essentially concentrated on two questions. Firstly, we have studied the relevance of erythrocyte gene repression by v-*erb*A for the leukemic phenotype, re-expressing v-*erb*A arrested genes by means of retroviral vectors (H. Beug and I. Leitner). And secondly, we have sought (and in part succeeded) to identify normal functions of the proto-oncogenes c-*erb*B (EGF/TGF*a* receptor) and c-*erb*A (thyroid hormone receptor *a*) and releated receptors in the proliferation and differentiation of nontransformed erythroid progenitors (Christian Schroeder and Leonie Gibson). In addition we have collaborated with the group of Martin Zenke with regard to the question, how c- and v-*erb*A repress and activate genes and how the activity of the c-*erb*A protein is regulated by phosphorylation (see research report Zenke).

Apart from this ongoing work on the AEV system, we are pursuing several related and newly initiated projects. Firstly we employ the conditional oncogene approach to express additional genes in erythroid cells. This "conditional concogene approach" consists of transforming erythroid progenitors with a transforming protein, the function of which can be switched on and off, e. g. a thermo-sensitive version of the tyrosine kinase oncoprotein v-*sea* (ts-v-*sea*). In its "on"-version, (i. e. at the permissive temperature, 37°C) the ts-v-*sea* oncoprotein allows to expand the transformed cells to suitable numbers. Normal red cell differentiation can then be induced by "switching off" oncoprotein function by simple shift to the nonpermissive temperature, 42°C.

Since a major drawback of the avian system is the lack of molecularly cloned hematopoietic growth factors (mammalian factors are inactive on avian cells) we are expressing mammalian receptors for such growth factors (Erythropoietin-(EPO)-receptor, GM-CSF-receptor) to render the cells dependent on cloned mammalian growth factors. This approach has been quite successful in case of the EPO receptor (Peter Steinlein, Oliver Wessely). Secondly, we are introducing ligand-dependent transcription factors (Estrogen-receptor, retinoic acid receptor) and fusion proteins between nuclear oncogenes (c-*myc*, c-*myb*, v-*jun* and v-*rel*) and the estrogen-receptor hormone binding domain (ER) rendering these oncoproteins functionally dependent on hormone into conditional oncogene-transformed erythroblasts to learn how these transcription factors might affect erythroid differentiation.

Two such hormone-regulated oncogene-steroid receptor fusion proteins (c-*fos* ER, c-*myc* ER) are turning out to be very useful to analyse processes important for carcinoma formation. Such processes are alterations in cell polarity, loss of differentiated functions and changes in epithelial/endothelial or cell-matrix interactions. These are being studied in a mammary epithelial cell system which retains many features of primary mammary gland cells. (E. Reichmann).

Finally, we have sought to analyse thyroid hormone receptor (c-*erb*A) function in neuronal cells, where this hormone receptor plays a crucial role *in vivo* during cell differentiation and cell function. C-*erb*A and its oncogenic version v-*erb*A were introduced into two neuronal cell lines to study differentiation regulation by these receptors at the molecular level (Alberto Munoz).

A. c- and v-*erb*A

Significance of v-erbA-induced gene repression for the leukemic phenotype: Reintroduction of the Band 3 and CA II genes into v-erbA -expressing erythroblasts

Irene Leitner, Christian Schroeder, Hartmut Beug in collaboration with Heinz Schwarz, Tübingen, and Sally Fuerstenberg and Björn 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. V-*erb*A affects erythroid cells in two ways. It causes an arrest in terminal differentiation and induces a profound change in erythroblast growth requirements, enabling v-*erb*A erythroblasts to grow in standard media. In contrast, cells lacking v-*erb*A require special growth conditions (Kahn et al,1986). To clearly define the role of band 3 and carbonic anhydrase gene repression in leukemia we have constructed retroviruses suitable for high expression of complete avian band 3 and CAII cDNAs in cells (see report 1990). The band 3 and CAII genes were then introduced into v-*erb*A expressing erythroblasts. While control ts-v-*sea*-v-*erb*A erythroblasts failed to express exogeneous Band 3, grew in standard tissue culture media and were tightly arrested in differentiation, erythroblasts expressing both v-*erb*A and exogenous Band 3 behaved like cells lacking v-*erb*A. They were unable to grow in standard media, developing large vacuoles before desintegrating under these conditions but grew normally in media suitable for growth of erythroblasts transformed by kinase oncogenes only. The same result was obtained with a v-*erb*A ts-v *erb*B expressing cell line (HD3) after expression of exogenous Band 3. In this cell line, we could show by immunofluorescence analysis of semithin Lowicryl sections (H. Schwarz) that the exogenously overexpressed band 3 protein was exclusively localized at the plasma membrane but missing in the vacuole membrane, ruling out toxic effects due to inappropriate localization. In contrast, Band 3 re-expression was unable to relieve the differentiation arrest caused by v-erbA in cells after shut off of the primary transforming kinase oncogene. Our results thus 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 but not for another aspect of leukemic transformation, that is, the arrest of differentiation.

Similar experiments were performed with the carbonic anhydrase gene. Since the activity of CA II is reduced but not completely blocked in v-*erb*A-containing erythroblasts, it was necessary to distinguish between endogenous and exogenous CA II at the protein level. We therefore fused the carbonic anhydrase cDNA to viral gag sequences and inserted it in two different retrovirus vectors that allowed high level expression of the gag-CA II protein in fibroblasts with an apparently unchanged enzyme activity. Although trials to introduce the gag-CA II gene in v-*erb*A-containing erythroblasts were hampered by its apparent toxicity in these cells, two clones stably expressing gag CAII at about 1/3 of the activity in erythrocytes could be obtained. These cells again were unable to proliferate in standard media, but grew with reduced rate in special media used for growth of erythroblasts without v-*erb*A. Furthermore, CAII seemed to partially overcome the v-*erb*A-induced differentiation arrest, although it is possible that this is a trivial effect due to seletive toxicity to immature cells.

In conclusion, repression of Band 3 and CAII are clearly responsible for part of the v-*erb*A-induced leukemic phenotype. Both genes, however, seem to play only a minor role, if any, in the v-*erb*A induced differentiation arrest.

Normal function of the thyroid hormone receptor a (c-erbA) and related receptors in regulation of erythroid differentiation

Mechanisms of how v-*erb*A inhibits erythroid differentiation and constitutively represses transcription of certain erythrocyte genes have been elucidated to some detail (see report 1990 and Zenke, 1991). Much less is known, however, about normal functions of the c-*erb*A proto-oncogene (thyroid hormone receptor *a*; TR*a*) in erythroid cell proliferation and differentiation. Recently we have found indications for such a normal pathway of c-*erb*A/TR*a* function that may well be a target for v-*erb*A oncogene action. We demonstrated that c-*erb*A/TR*a* and, even more strikingly, the retinoic acid receptor (RAR*a*), modulated differentiation of various normal or quasi-normal erythroid progenitors if stimulated by the respective ligands, thyroid hormone (T3) and retinoic acid (RA). When RA was added pulsewise to immature erythroid progenitors, differentiation was accelerated while more mature cells underwent premature cell death. Thyroid hormone (T3) alone caused similar, but weaker effects, most likely due to the very low expression of this receptor in the cells available *in vitro*. Interestingly, T3 strongly enhanced the action of RA, suggesting cooperative action of the two receptors in modulating erythroid differentiation.

Expression of the human RAR*a* in receptor-negative erythroblasts conferred RA-induced regulation of differentiation to the otherwise unresponsive cells, thus showing that the RAR*a* is essential for the RA effect. Likewise, enhanced expression of exogenous c-*erb*A/TR*a* in erythroblasts rendered them highly susceptible to modulation of differentiation by T3, suggesting a similar function of both receptors.

Oncogene cooperation between kinase oncogenes and v-erbA is required to prevent differentiation induction by normal, endogenous c-erbA/TRa and RARa

In a continuation of the work above we have studied if and to what extent the normal regulation of differentiation by endogenous nuclear hormone receptors may interfere with transformation of erythroblasts by tyrosine kinases, by v-*erb*A, or by a combination of these oncogenes. The endogenous RAR*a* efficiently induced terminal differentiation in erythroblasts transformed by several tyrosine kinase oncogenes, thus abolishing their main transforming effect, i.e. self-renewal induction. The same is true for c-*erb*A/TR*a*, if expressed at sufficient levels by a c-*erb*A/TRa expressing retrovirus. Both RAR*a* and overexpressed c-*erb*A/TR*a* can also revert the differentiation arrest induced by v-*erb*A in the absence of a second oncogene. However, the same receptors are completely unable to affect transformation and induce differentiation in erythroblasts, doubly transformed by tyrosine kinases and v-*erb*A. These results suggest that oncogene cooperation between kinase oncogenes and v-*erb*A is required to protect leukemic erythroblasts from differentiation induction via endogenous, nuclear hormone receptors.

Endogenous c-*erb*A/TR*a* and RAR*a* apparently co-operated in abolishing erythroblast self-renewal and inducing differentiation, since the respective ligands acted in a synergistic fashion and overexpressed, nonliganded c-*erb*A/TR*a* suppressed endogenous RAR*a* function in differentiation induction. By studying various c/v-*erb*A chimeric proteins for their ability to quench the retinoic acid response in erythroblasts, we could show that the functional co-operation between *erb*A/TR*a* and RAR*a* requires the receptor dimerization domain, suggesting that TR*a*/RAR*a* heterodimers play a role in the regulation of erythroid differentiation.

Future analysis will focus on cells expressing exogenous c-*erb*A/TR*a* and RAR*a* as well as on TR-v-*erb*A chimeras and dominant negative RAR versions (available from M. Karin, San Diego) with the main aim to identify possible oncogenic RAR versions.

Functional significance of c-erbA-phosphorylation

Hartmut Beug and Martin Zenke, in collaboration with Corinne Glineur and Jaques 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 1990, we completed the functional analysis of mutant v-*erb*A proteins in which these phosphorylate serines had been converted to alanines. We showed that lack of v-*erb*A-phosphorylation essentially abolished all biological activities of v-*erb*A i.e arrest of erythroid differentiation, alterations of *in-vitro* medium requirements and suppression of erythrocyte-specific gene transcription. Similarly, inhibition of *in-vivo* phosphorylation by the protein kinase inhibitor H7 led to almost complete loss of v-*erb*A function in all these parameters.

In 1991, we have initiated similar studies to analyse the significance of phosphorylation of the c-*erb*A/thyroid hormone receptor *a* for its function in erythroblasts, concentrating both on the Ser 28/29 sites also present in v-*erb*A and on a highly conserved casein kinase II site at Ser 12. The respective vectors containing c-*erb*A, both as fusion proteins with viral *gag* and as bona fide c-*erb*A proteins have been made and tested for their ability to repress and transactivate via a thyroid hormone response element (TRE) in transient transcription assays (using a transfectable erythroblast line, see report Zenke 1991). All mutants were capable of transactivation while the ALA 28/29 mutant was probably somewhat defective in repression. Work to characterize these mutants in transformed erythroblasts is currently in progress.

Function of thyroid hormone receptor a (c-erbA) and its oncogenic version v-erbA in neuronal and chromaffine differentiation of the adrenal gland progenitor cell line PC12

Alberto Munoz and Hartmut Beug

One of the most important *in vivo* functions of the thyroid hormone receptor (c-*erb*A) is its involvement in differentiation and function of neuronal cells. To develop an *in vitro* cell system in which these functions of c-*erb*A/TR*a* could be studied at the molecular level, c-*erb*A and its oncogenic variant v-*erb*A were introduced into PC 12 cells, a cell line in which aspects of neuronal and chromaffine cell differentiation can be induced by nerve growth factor (NGF) and dexamethasone (DEX), resepctively. In the absence of a ligand, c-*erb*A/TR*a* suppressed NGF-induced neurite outgrowth and repressed transcription of a number of NGF-induced genes. Addition of T3 to the NGF-induced c-*erb*A-PC-12 cells relieved this differentiation block, allowing normal NGF-dependent differentiation and normal or even enhanced expression of NGF-dependent genes. Interestingly the ligand-activated c-*erb*A/TR*a* also rendered the PC-12 cells dependent on NGF for survival and growth, perhaps mimicking the *in vivo* action of NGF as a trophic factor.

In line with the idea, that c-*erb*A induces "commitment" of PC 12 cells to the neuronal pathway, c-*erb*A arrested chromaffine differentiation. It prevented the formation of dense, epitheloid islands induced by DEX in uninfected PC 12 cells and suppressed or reduced expression of chromaffine marker genes (such as tyrosine hydroxylase) as well as general epithelial markers (cytokeratins, N-cadherin) These c-*erb*A/TR*a* effects occurred both in presence and in absence of the ligand (T3) but were somewhat more prominent in absence of the ligand.

As expected, the effects caused by v-*erb*A on PC 12 differentiation were independent of ligand and somewhat aberrant. V-*erb*A-expressing PC 12 cells failed to efficiently differentiate in response to NGF. Some but not all NGF-induced genes were constitutively repressed by v-*erb*A. Surprisingly, some chromaffine marker genes were constitutively overexpressed in the v-*erb*A-PC12 cells, consistent with the notion that v-*erb*A causes an aberrant "interlineage" phenotype in PC12 cells.

B. Conditionally transformed avian hematopoietic cells as models to functionally analyse mammalian hematopoietic growth factor receptors

One of the main reasons, why avian hematopoietic cells are largely useless as a model to study the regulation of hematopoiesis by cytokines is that mammalian growth factors do not function in avian cells and that (with one exception) avian growth factors or receptors are not available in pure or even in a molecular cloned form. On the other hand, primary cell strains of hematopoietic cells transformed by conditional oncogenes that can be expanded in presence of a functional oncogene but undergo normal terminal differentiation and respond normally to cytokines after switching off oncogene function are so far only available in the avian system. We therefore have initiated studies, whether mammalian receptors for hematopoietic growth factors would function normally in avian cells, hoping that such a result would allow us to study cytokine action in normal and leukemic avian hematopoietic cells much more easily.

Functional expression of the mammalian erythropoietin receptor in differentiating avian erythroid cells

Peter Steinlein and Hartmut Beug

Transformation of avian erythroid cells by tyrosine-kinase oncogenes comprises two major changes induced in erythroid progenitors: induction of self-renewal and abrogation of their dependence on hematopoietic growth factors, particularly erythropoietin (EPO). This latter oncogene effect was practically resistant to molecular analysis since avian cells do not respond to mammalian EPO or other hematopoietic growth factors. Furthermore, avian EPO or its receptor seem difficult if not impossible to clone.

We decided to circumvent this problem by introducing the murine EPO-receptor (mEPO-R) into avian hematopoietic cells. For this a suitable avian retrovirus vector was constructed and used to infect kinase oncogene-transformed erythroblasts. The exogenously expressed mEPO-R was present at physiological expression levels (200–1000 receptors/cell) exhibiting a single class of high affinity binding sites (kD: 100–180 pM). The mammalian mEPO-R was also fully functional. When mEPO expressing erythroblasts transformed by a conditional kinase oncogene were induced to differentiate after switching off oncogene function, human recombinant EPO (hrEPO) was indistinguishable from partially purified chicken EPO in its effects on red cell differentiation. This was true for proliferation kinetics, viability, accumulation of hemoglobin, ability to form CFU-E-like colonies and morphological and histochemical aspects of red cell differentiation. As expected, control cells devoid of the mammalian EPO-R showed no detecable response to hrEPO.

The mEPO-R also co-operated with endogenous avian tyrosine kinase receptors such as the insulin receptor. In mEPO-R expressing cells, insulin synergized with both avian and mammalian EPO in enhancing cell viability and erythrocyte yield. In contrast, insulin growth factor 1 (IGF-1), reported to exert this effect in mammalian erythroid cells had no effect.

From these results, we conclude that the ectopically expressed murine EPO-R uses the signal transduction machinery of avian erythroblasts the same way the native receptor does and co-operates with endogenous avian growth factor receptors. We intend to use this approach to elucidate how signal transduction by tyrosine kinase receptors and nonkinase receptors (such as EPO-R) do interplay and how oncogenic activation of the avian c-*erb*B/TGF*a* receptor can bypass EPO receptor function in kinase oncogene transformed cells.

Expression of the GM-CSF receptor in avian hematopoietic cells

Oliver Wessely, Hartmut Beug and Peter Steinlein

It is unclear, whether the chicken hematopoietic system utilizes multilineage growth factors like GM-CSF and Il 3, since the respective mammalian factors are inactive in the chicken and avian activities with a similar target cell spectrum are unknown. We therefore constructed an avian retrovirus expressing the *a* chain of the murine GM-CSF receptor. The receptor was successfully expressed in fibroblasts. Trials to introduce it into avian erythroid (ts -v-*sea*, TGF*a* dependent erythroblasts) myeloid (ts myb transformed myeloblasts) and lymphoid cells (*rel*-ER-transformed lymphoblasts, see report Zenke 1991) are currently in progress.

C. Ligand-dependent transcription factors in erythroid differentiation

Significance of estrogen receptor expression and -function in normal erythroid progenitors.

Christian Schroeder, Leonie Gibson and Hartmut Beug

During a screen of differentiating ts-oncogene transformed erythroblasts for their possible response to steroid hormones, estrogen was found to have an unexpectedly strong activity, consisting of stimulation proliferation of the differentiating cells and delaying their terminal maturation. We therefore tried to demonstrate the presence of the estrogen receptor (ER) in normal and transformed avian erythroid cells and its possible role in regulation of normal erythroid differentiation. These studies are still in progress.

D. Carcinogenesis

Conditional versions of nuclear oncogenes as tools to study epithelial cell transformation in vitro

Ernst Reichmann, Heinz Schwarz, Meinrad Busslinger and Hartmut Beug

In 1991, we introduced a novel *in vitro* approach to study, how oncogenes affect epithelial cell polarity and differentiation. This approach consists of introducing conditional ("switch on or off") oncogenes into highly polarizing mammary epithelial cells. With the oncogene switched **off,** a fully polarized epithelial cell layer is allowed to develop. Potential oncogene effects on the epithelial cell phenotype can then be studied after switching **on** oncogene function.

Hybrid c-*fos*- and c-*myc*-estrogen receptor fusion proteins (c-*fos*ER and c-*myc*ER, (Superti-Furga et al., 1991; Eilers et al., 1989)) were introduced into fibroblasts using suitable retroviral vectors and shown to cause hormone-dependent transformation. C-*fos*ER and c-*myc*ER were then introduced into mammary epithelial cell lines that highly polarize and exhibit distinct mammary gland-specific differentiation properties when cultivated on a permeable support (Reichmann et al, 1989). In 1991, we have analysed the biological properties of these c-*myc*ER and c-*fos*ER epithelial cells to some detail.

In their ligand-activated state, c-*fos*ER and c-*myc*ER caused oncogene-specific alterations in the epithelial cell clones. Estrogen (E2)-dependent c-*myc*ER activation caused the cells to grow to abnormally high cell densities, but did not detectably affect epithelial cell polarity. In contrast, hormone-induced activation of c-*Fos*ER had much more drastic effects. Activation of c-*Fos*ER for time periods shorter than 3 hours caused the reversible loss of cell polarity, as indicated by formation of irregular, sometimes multilayered cell sheets and decrease in transepithelial resistance. We are currently trying to determine if sorting of apical (aminopeptidase, retroviruses) and baso-lateral markers (Uvomorulin) is reversibly affected by short-term activated c-*fos* ER. Also ultrastructural studies, (which already indicate that activated c-*fos* ER abolishes tight junctions, but does not disrupt desmosomes) are in progress to determine to what extent the c-*fos*ER disruption of an organized epithelial cell layer is reversible.

Ligand activation of the c-*fos*ER oncogene for longer time periods resulted in the irreversible loss of polar organisation and in the formation of multilayered cell sheets, consisting of cells which exhibited a fibroblastic morphology. These cells no longer expressed epithelial marker proteins such as uvomorulin, tight junction protein (ZO-1) or desmoplakin, but instead expressed mesenchymal markers such as vimentin and fibronectin. At least some of these changes occur at the transcriptional level as shown by respective northern blot analysis. The cells, however, also retained epthelial markers such as cytokeratins and a dense layer of surface microvilli typical for the apical surfaces of epithelial cells.

Our experiments indicate that the c-*fos*ER oncogene can induce both reversible and irreversible changes of epithelial cell polarity. The irreversible changes closely resemble those occurring during epithelial-mesenchymal transformation, a process occurring at certain steps of normal development. We expect this new *in vitro* system to be useful to study both normal biogenesis of epithelial cell polarity and the importance of oncogene-induced disruption of polarity and induction of a mesenchymal pattern of gene expression in carcinogenesis.

PUBLICATIONS SUBMITTED 1991

1. *Disela, Ch., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., and Zenke, M. (1991): v-erbA overexpresison is required to extinguish c-erbA function in erythroid cell differentiation and regulation of the erbA target gene CAII. Genes & Development* **5**, *2033–2047.*
2. *Schroeder, Ch., Gibson, L., Zenke, M., and Beug, H. (1991): Modulation of normal erythroid differentiation by the endogenous thyroid hormone- and retinoic acid receptors: a possible target for v-erbA oncogene action. Oncogene, in press.*
3. *Schroeder, Ch., Gibson, L., and Beug, H. (1991): The v-erbA oncogene requires cooperation with tyrosine kinases to arrest erythroid differentiation induced by ligand activated endogenous c-erbA and retinoic acid receptors. Oncogene, in press.*
4. *Beug, H., Doederlein, G., and Zenke, M. (1991): Transformation by v-erbA and v-erbB oncogenes: Independent modulation of differentiation and proliferation in erythroid progenitors. In Bristol Myers Symposia on Nuclear Processes and Oncogenes, Vol. 15 (P.A. Sharp, ed.). Academic Press Inc., Orlando, Florida, in press.*
5. *Morrison, L. E., Boehmelt, G., Beug, H. and Enrietto, P. (1991): Expression of v-rel in a replication-competent virus: Transformation and biochemical characterization. Oncogene* **6**, *1657–1666.*
6. *Kennedy, M., Beug, H., Wagner, E. F., and Keller, G. (1991): Factor dependent erythroid cell lines derived from mice transplanted with hematopoietic cells expressing the v-src oncogene. Blood, in press.*
7. *Ulrich, E., Boehmelt, G., Bird, A., and Beug, H. (1991): Immortalization of conditonally transformed chicken cells: Loss of normal p53 expression is an early step that is independent of cell transformation. Genes & Dev., submitted.*
8. *Ghysdael, J., and Beug, H. (1991): The leukemia oncogene v-erbA: A dominant negative version of a ligand-dependent transcription factor that regulates red cell differentiation. Cancer Surveys, in press.*

OTHER REFERENCES

Reichmann et al. (1989): J. Cell Biol. **109**, *1127–1138.*

Superti-Furga, G., Bergers, G., Picard, D., and Busslinger, M. (1991): Proc. Natl. Acad. Sci. USA **88**, *5114–5118.*

Kahn et al. (1986): In XII Sympos. for Comp. Res. on Leukemia and related diseases. F. Deinhard ed. Springer Verlag, Heidelberg, pp. 41–50.

Eilers, M., Picard, D., Yamamoto, K. R. and Bishop, M. (1989): Nature **340**, *66–69.*

Goldberg, Y., Glineur, C., Gesquiere, J. C., Ricouart, A., Sap, J., Vennström, B., and Ghysdael, J. (1988) EMBO J. 7, 2425–2433.

Transcription factors involved in signal transduction and differentiation

Meinrad BUSSLINGER	Senior scientist
Shirley WANG	Postdoc
Ben ADAMS	Postdoc
Christopher WRIGHTON	Postdoc
Zbynek KOZMIK	Postdoc
Sylvia BRASELMANN	PhD-student
Gabriele BERGERS	PhD-student
Petra DÖRFLER	PhD-student
Heinz ULLY	PhD-student
Paula GRANINGER	Laboratory technician
Pavel URBANEK	Visiting scientist

1) The role of the oncoprotein Fos in signal transduction

Introduction

The proto-oncogene c-*fos* is thought to play a central role in signal transduction by coupling short-term stimulation of a cell to long-term alterations in gene expression. The c-*fos* gene codes for a nuclear protein that is an important component of the transcription factor AP-1. In the AP-1 complex the Fos protein is bound to the product of another proto-oncogene, c-*jun*, via a specific leucine zipper interaction. The different domains of the Fos protein which are responsible for AP-1 dependent transactivation and transformation of fibroblasts have been mapped in great detail. In contrast, the relevant endogenous Fos target genes, that are involved in the control of proliferation and transformation, are still largely unknown. We have taken advantage of the highly selective posttranslational Fos-ER inductionsystem to search for such Fos target genes (Superti-Furga et al., 1991, see Figure 1). During the last year we have identified and characterized three Fos-regulated genes coding for Fit-1, Fra-1 and ornithine decarboxylase (ODC).

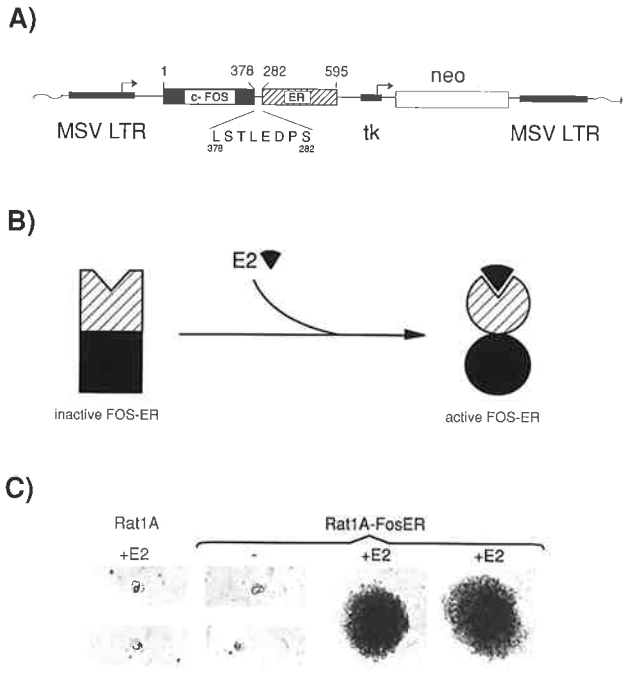


Figure 1. *The Fos-ER induction system. A) Mouse c-fos cDNA was linked at the C-terminus to cDNA encoding the hormone-binding domain of the human estrogen receptor (ER). This chimaeric gene was constitutively expressed from the Moloney murine sarcoma virus long terminal repeat (MSV LTR) in stable transfectants of rat fibroblasts or PC12 cells. B) Principle of the posttranslational induction system. The Fos-ER protein is expressed to high levels within a given cell, but it is inactive in the absence of estrogen (E2). C) Hormone-dependent transformation of rat fibroblasts (Rat-1A) by Fos-ER. Fos-ER expressing cells are only able to grow in soft agar in the presence of estrogen. For details see Superti-Furga et al. 1991.*

Characterization of Fos target genes

Gabriele Bergers, Sylvia Braselmann, Paula Graninger

The Fit-1 gene was isolated by differential screening of a subtracted cDNA library which was established from estrogen-induced rat fibroblasts. Transcription of the Fit-1 gene is rapidly stimulated by ligand-activated Fos-ER even in the presence of protein synthesis inhibitors suggesting that Fos activity directly regulates the Fit-1 gene promoter. In agreement with this finding, serum induction of the Fit-1 gene is strictly dependent on the synthesis of immediate early gene products such as c-Fos. The promoter of the Fit-1 gene has been cloned and was shown to contain regulatory elements that mediate transcriptional induction by c-Fos and by serum. Overexpression of the Fit-1 gene is apparently not sufficient for morphological transformation of rat fibroblasts. The Fit-1 mRNA appears, however, to be the most abundant Fos-inducible transcript in these cells, because it has been isolated as independent cDNA clones many times from a subtracted cDNA library. Sequence comparison revealed that the Fit-1 gene is the rat homologue of the mouse T1 gene which has previously been identified as a Ras-inducible gene in NIH 3T3 fibroblasts (Klemenz et al., 1989). Both genes belong to the immunoglobulin gene superfamily and their proteins show the highest homology with the human carcinoembryonic antigen.

Three Jun-related proteins (c-Jun, JunB and JunD) and four Fos-related proteins (c-Fos, FosB, Fra-1 and Fra-2) contribute to the heterogeneous composition of the transcription factor AP-1. We have analyzed the expression of the genes coding for these AP-1 proteins in Fos-ER expressing fibroblasts. Estrogen was able to induce the transcription of only the *fra*-1 gene. This crosstalk between c-Fos and the *fra*-1 gene appears to be a general phenomenon as it is also seen in other cell types. Fra-1 was shown to be a more weakly transforming oncoprotein than c-Fos, as ectopic expression of the fra-1 gene in rat fibroblasts promoted growth in soft agar, but did not result in overt morphological transformation. It is therefore conceivable that Fos-induced expression of the *fra*-1 gene is responsible for part of the Fos transformation phenotype of fibroblasts.

Uncoupled c-Fos activity in PC12 pheochromocytoma cells: short and long term effects

Christopher Wrighton, Heinz Ully

Constitutive expression of unliganded Fos-ER fusion protein in a PC12 cell background has no apparent effect on gene expression in either growing or NGF-treated cells. Addition of estrogen, however, rapidly increases the steady state mRNA level of at least three genes, *fra*-1, tyrosine hydroxylase and ornithine decarboxylase (ODC). In the latter case this increase is clearly a direct effect of Fos-ER involving DNA binding since it is cycloheximide insensitive, depends on an intact DNA binding domain of the Fos-ER protein and is regulated at the level of transcription initiation. It is likely that Fos induction of the ODC gene involves one or both AP-1 sites present in the ODC gene. However, the ODC gene is not inducible in a Fos-ER expressing FR3T3 fibroblast cell line and hence the activity of Fos-ER is cell background specific with interesting implications for possible mechanisms underlying signal transduction specificity (Wrighton and Busslinger, 1991).

In contrast to the above, prolonged Fos-ER activation has been found to strongly repress both basal and inducible (NGF or dexamethasone) expression of all genes so far analyzed. In the case of NGF, this general repression effect correlates well with an observed "block" of the neuronal differentiation pathway. Most notably, Fos-ER represses the putative neuronal determination gene Mash-1. However, Fos-ER activation in cycling cells itself generates a differentiated phenotype that resembles morphologically that induced by dexamethasone, an agent that drives PC12 cells to a more mature chromaffin phenotype.

Uncoupled c-Fos activity clearly has dual effects in PC12 cells, in the short term inducing expression of at least three genes whilst in the long term generally repressing gene expression. The link between the latter effect and profound effects on PC12 differentiation strongly suggests that Fos-ER may interfere with key regulatory molecules involved in the neuron versus chromaffin cell decision. We have thus in effect a conditional PC12 mutant that is a potentially powerful tool with which to identify these important molecules.

2) Characterization of the B-cell-specific transcription factor BSAP

Introduction

We have previously identified the B-cell-specific transcription factor BSAP as a mammalian homologue of the sea urchin protein TSAP, which is responsible for the developmental and tissue-specific regulation of two non-allelic pairs of late histone H2A-2 and H2B-2 genes (Barberis et al. 1989). The mammalian factor BSAP interacts with the four TSAP-binding sites present in these histone gene promoters in a manner that is indistinguishable from the sea urchin protein. BSAP is exclusively expressed in the B-lymphoid lineage within the hematopoietic system. Equal BSAP-binding activity is observed in pro-B, pre-B and mature B cells, while it is undetectable in terminally differentiated plasma cells. BSAP was shown to act as a positive transcription factor on an artificial promoter containing a sea urchin TSAP-binding site. The strict B-cell lineage fidelity of BSAP suggests an important role of this transcription factor in B-cell ontogeny. However, known regulatory regions of immunoglobulin and class II MHC genes lack high affinity BSAP-binding sites suggesting that BSAP regulates a different set of B-lymphoid-specific genes (Barberis et al. 1990). During the last year we have identified and characterized the CD19 gene as one of the BSAP target genes. Moreover, we have biochemically purified BSAP to homogeneity followed by protein sequencing and cDNA cloning.

The gene coding for the B-cell surface marker CD19 is regulated by BSAP

Zbynek Kozmik, Shirley Wang, Petra Dörfler

The CD19 protein is expressed on the surface of all B-lymphoid cells with the exception of terminally differentiated plasma cells and has been implicated as a signal-transducing receptor in the control of proliferation and differentiation (Nadler et al, 1983). We have demonstrated complete correlation between the expression pattern of the CD19 gene and the B-cell-specific transcription factor BSAP in a large panel of B-lymphoid cell lines. The human CD19 gene has been cloned and several BSAP-binding sites have been mapped to 5' and 3' flanking sequences and to the fourth intron by in vitro protein-DNA binding studies. In particular, a high affinity BSAP-binding site instead of a TATA sequence was located in the –30 promoter region upstream of a cluster of heterogeneous transcription start sites. *In vivo* footprinting experiments demonstrated that this site is occupied by BSAP in a CD19 expressing B-cell line, but not in plasma or HeLa cells. This high affinity site has been conserved in the CD19 promoter between human and mouse and was furthermore shown to confer B-cell specificity to a β-globin reporter gene in transient transfection experiments. In addition, BSAP was shown to be the only abundant DNA-binding activity of B-cell nuclear extracts that interacts with the CD19 promoter. Taken together all this evidence strongly implicates BSAP in the regulation of the CD19 gene (Kozmik et al., 1991).

cDNA cloning of the B-cell-specific transcription factor BSAP

Ben Adams, Petra Dörfler, Pavel Urbanek

BSAP consists of a single 50 kDa polypeptide which was purified to homogeneity from human BJA-B cells by wheat germ lectin and DNA affinity chromatography followed by HPLC fractionation and protein sequencing (in collaboration with Dr. I. Maurer-Fogy, Bender, Vienna). Two reliable peptide sequences were obtained and subsequently used to design specific primers for PCR cloning of BSAP cDNA from BJA-B cells. Northern blot analysis with this cDNA probe revealed that BSAP transcripts are present in pro-B, pre-B and mature B cells, but not in plasma cells suggesting that BSAP gene expression is mainly regulated at the transcriptional level. A 3.3 kb cDNA clone was subsequently isolated which gave rise to intact BSAP binding activity in transiently transfected NIH 3T3 cells and hence contained the entire coding region for the BSAP protein. DNA sequencing and in vitro mutagenesis of the BSAP cDNA are in progress to define the functional protein domains involved in DNA binding and transcriptional activation. The chromosomal BSAP gene has been cloned and will be used to disrupt the endogenous BSAP gene by homologous recombination in transgenic mice. These loss-of-function experiments should allow us to address the question of how important BSAP is for B-cell differentiation and possibly other developmental processes.

PUBLICATIONS SUBMITTED 1991

Superti-Furga, G., Bergers, G., Picard, D., and Busslinger, M. (1991): Hormone-dependent transcriptional regulation and cellular transformation by Fos-steroid receptor fusion proteins. Proc. Natl. Acad. Sci. USA ***88****, 5114–5118.*

Kozmik, Z., Wang, S., Dörfler, P., Adams, B., and Busslinger, M. (1991): The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. Mol. Cell. Biol., submitted.

Wrighton, C., and Busslinger, M. (1991): c-Fos activity is sufficient to stimulate transcription of the ornithine decarboxylase gene in PC12 cells, but not in fibroblasts. Mol. Cell. Biol., submitted.

OTHER REFERENCES

Barberis, A., Superti-Furga, G., Vitelli, L., Kemler, I., and Busslinger, M. (1989): Genes Dev. ***3****, 663–675.*

Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1990): Genes Dev. ***4****, 849–859.*

Klemenz, R., Hoffmann, S., and Werenskiold, A.-K. (1989): Proc. Natl. Acad. Sci. USA ***86****, 5708–5712.*

Nadler, L. M., Anderson, K. C., Marti, G., Bates, M., Park, E., Daley, J. F., and Schlossman, S. F. (1983): J. Immunol. ***131****, 244–250.*

Cell Cycle Control in Yeast

Kim NASMYTH	Senior scientist
David LYDALL	Postdocs
Uttam SURANA	
Angelika AMON	PhD-students
Léon DIRICK	
Thomas MOLL	
Rita TABA	
Fatima CVRCKOVA	
Herbert AUER	Laboratory technician

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

Léon Dirick, Thomas Moll, Rita Taba, Herbert Auer and Kim Nasmyth

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 that 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, 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 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. *CLN1* and *CLN2* transcripts appear only transiently as cells undergo START. What then regulates the activity of these *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. Several lines of evidence suggest that the essential role of *SWI4* and *SWI6* is to activate GI cyclin genes: 1) *CLN1* and *CLN2* transcripts are not present in *swi4 swi6* double mutants; 2) A complex containing SWI4 and SWI6 proteins binds to $CACGA_4$ sequences within the *CLN2* promoter, 3) moderate ectopic expression of *CLN2* from the s. pombe ADH promoter restores the growth of *swi4 swi6* double mutants. Surprisingly, *CLN3* transcription is not dependent on *SWI4* or *SWI6* and yet its function is (see fig. 1). To explain this paradox we propose that in the absence of *CLN1* and *CLN2*, CLN3 needs an unknown factor (marked X in fig. 1) and that the transcription of X may be dependent on *SWI4* and *SWI6*.

A Paradox: CLN3 function is dependent on SWI 4,6 though its transcription is not.

Genotype	Growth phenotype	Conclusion
cln1Δ cln2Δ CLN3	+	G1 cyclin CLN3
cln1Δ cln2Δ cln3Δ	–	has a START function
swi4Δ swi6Δ	–	insufficient G1 cyclins are active in *swi4 swi6* double mutants
swi4Δ swi6Δ pADH - CLN2	+	
cln1Δ cln2Δ CLN3 swi4Δ	–	The START function of CLN3 is dependent on SWI4 and SWI6
cln1Δ cln2Δ CLN3 swi6Δ	–	

Hypothesis:

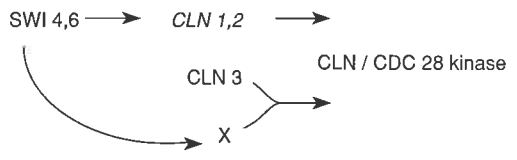
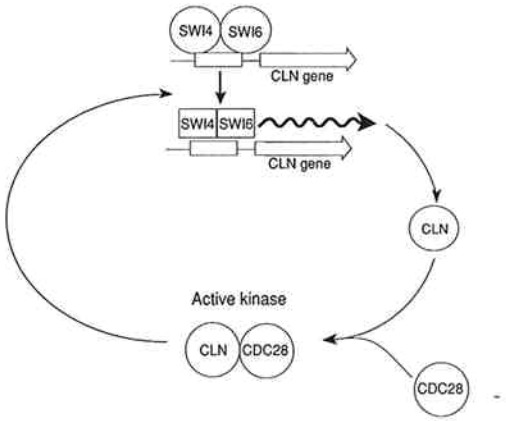


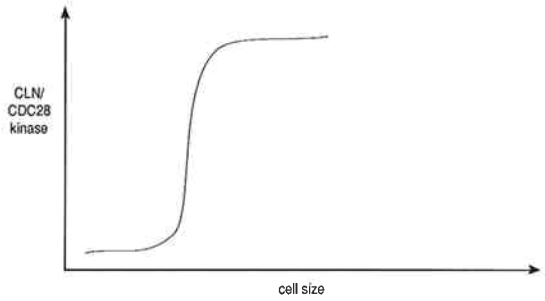
Figure 1

To explain how SWI4 and SWI6 function can be dependent upon the CDC28 kinase (as in the case of *HO*) even though they are activators (via the *CLN* genes) of CDC28, we propose that the full activation of the G1 form of the CDC28 kinase involves a positive feedback loop (see fig. 2). We have confirmed two crucial predictions of this hypothesis: first that the cell cycle dependent appearance of *CLN1* and *CLN2* RNAs should be *CDC28* dependent and then that the activation of one cyclin should cause the activation of others.

CLNs may promote their synthesis via SWI4,6



(+)ve feedback may facilitate all or none CLN/CDC28 kinase activation and hence irreversibility of START



How the switch from Low to high CLN expression is triggered by cell size is unknown.

Figure 2

Another early consequence of the passage of cells through START is the transcriptional activation of several genes required for DNA synthesis. In most of these genes, one or several copies of the sequence ACGCGT (the recognition site for the MluI restriction enzyme) is present in their promoter regions (see fig. 3). In the cases of the *TMP1* gene (encoding the enzyme thymidylate synthase) and *POL1* (coding for DNA polymerase) the MluI motif has been shown to be responsible for cell cycle dependent transcription. We now have evidence that a complex containing SWI6 and another protein of 120 kd distinct from SWI4 may bind the MluI motif. Thus, SWI6 may be a component of two different START dependent transcription complexes (see fig. 4)

START specific gene transcription

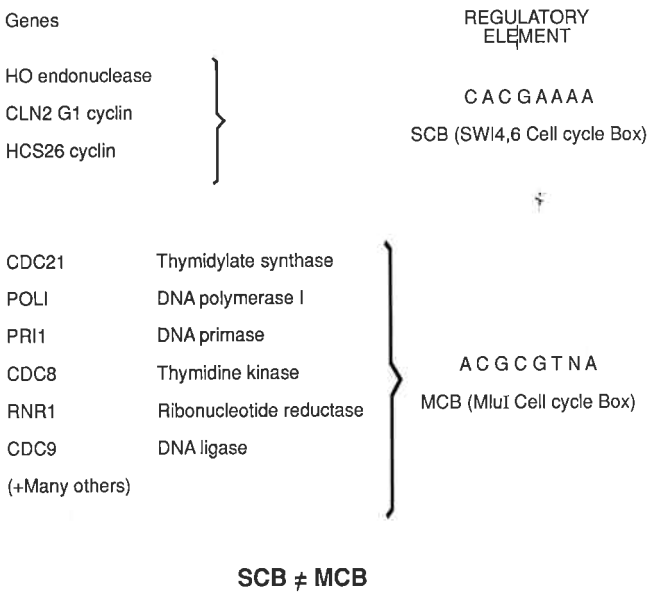


Figure 3

Transcriptional controls at START

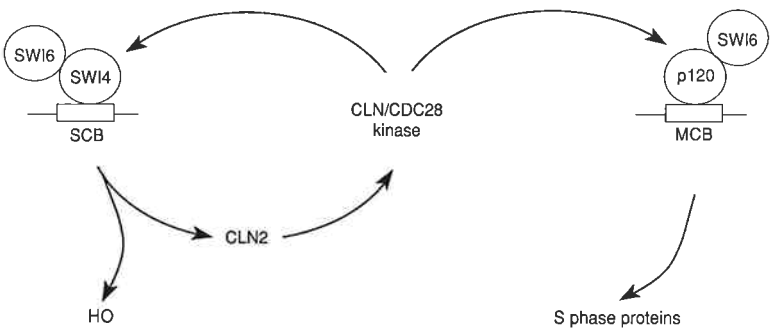


Figure 4

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 of the *SWI5* gene 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 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.

Genes required for G_1 cyclin activity in yeast

Fatima Cvrckova

In budding yeast, commitment to the mitotic cell cycle (START) is regulated by the Cdc28 protein kinase (see fig. 4). Putative regulatory subunits of this kinase, G_1 cyclins, are encoded by three functionally redundant genes *CLN1*, *CLN2*, *CLN3*. At least one of these genes has to be present to keep the cell alive and mutants lacking any two of them are viable in most genetic backgrounds. However, we have found that *cln1D cln2D* double mutants are inviable in the "wild type" W303 genetic background. Viability of *cln1D cln2D* cells depends on the allelic state of a single chromosomal gene (*CLA1* = Cln activator). The death of *cln1D cln2D cla1* cells seems to be caused by absence of active G_1 cyclins. This is consistent with the finding that these cells can be rescued by low-level constitutive expression of Cln2, but not by mild overexpression of Cln3 or its hyperactive form Cln3-1.

Recently polymorphism of the *SSD1/SRK1* gene has been reported to be responsible for some G_1-related phenotypes observed in W303. *SSD1/SRK1* is believed to be involved in protein phosphatase function and in the cAMP-mediated signal transduction presumably at the level of protein kinase A or downstream. We have now shown that *CLA1* is allelic to *SSD1*. Our finding that the *SSD1* gene (= *SRK1* = *CLA1*) may be involved also in activation of Cln3 might provide a link between the cAMP-signalling pathway and START regulation.

Further studies on the interaction of *SSD1/SRK1/CLA1* with the Cdc28/Cln complex as well as a search for other genes required for Cln3 activity are in progress.

Control of mitosis in Saccharomyces cerevisiae: the regulation of CDC28 kinase activity during progression through mitosis

Angelika Amon and Uttam Surana

In the budding yeast *S. cerevisiae* passage through cell cycle is regulated at the G1/S and G2/M transitions. Both transitions require the CDC28 protein kinase. We have previously reported the isolation of genes *(CLB)* encoding mitotic cyclins and have shown that the CDC28 activity presumably required for progression through the M-phase is dependent on the *CLB* gene products (Surana et al. Cell 65, 145–161; 1991). In *Xenopus* oocytes and *S. pombe* the activation of a similar mitotic kinase (MPF) containing the homologous protein cdc2 is achieved via dephosphorylation of a highly conserved Tyr15 residue. Substitution of the tyrosine residue by phenylalanine causes premature entry into mitosis suggesting that the phosphorylation state of Tyr15 is important for the regulated entry into mitosis. The activity of cdc2 kinase peaks at

metaphase and then sharply declines correlating with the transition to anaphase. Preventing the destruction of kinase activity leads to arrest in metaphase. This model for cdc 2 is shown in fig. 5.

To further our understanding of mitosis, we have asked if the budding yeast CDC28/CLB kinase is similarly regulated. We have examined the state of CDC28 phosphorylation and show that the Tyr19 residue of CDC28 (corresponding to Tyr15 of cdc2) is phosphorylated not only during G2 but also in S phase and undergoes dephosphorylation during mitosis. Surprisingly, however, its substitution by phenylalanine does not have any discernable phenotype with regard to the cell cycle progression implying that the dephosphorylation of Tyr19 may not be the prime regulatory event for the initiation of mitosis in budding yeast. We have also measured the CDC28/CLB2 kinase activity in *cdc15* mutants which undergo anaphase but are unable to complete mitosis at the restrictive temperature (37°C). In these mutants the CDC28/CLB2 dependent histone H1 kinase activity rises as cells reach metaphase but there is no drastic drop in the level of this activity as cells undergo anaphase indicating that in the budding yeast transition to anaphase occurs despite the presence of high H1 kinase activity (see fig. 6). The kinase activity does, however, decline rapidly when cells are released from the anaphase arrest. Therefore, it is the final exit from mitosis

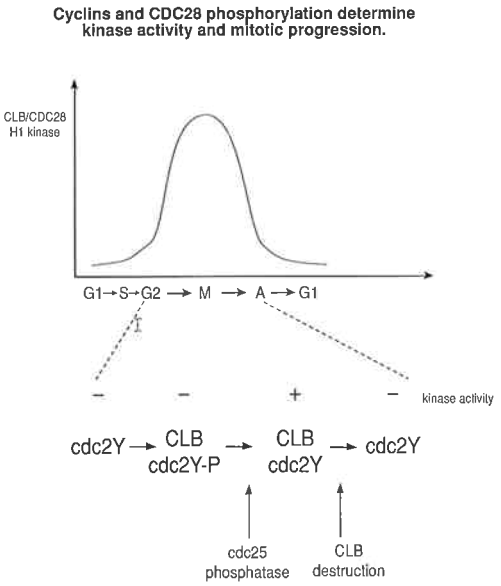


Figure 5

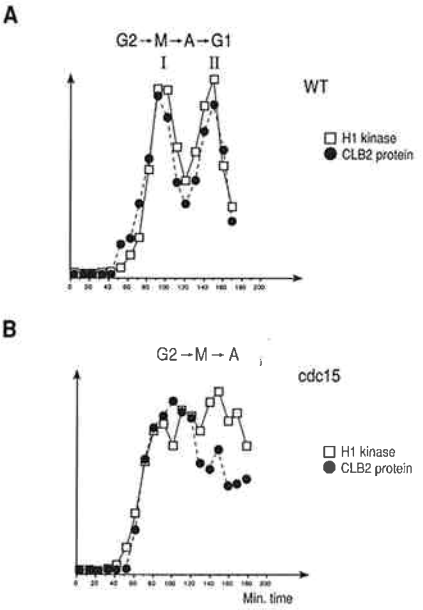


Figure 6

and not the metaphase to anaphase transition which is marked by the catastrophic destruction of the CDC28/CLB kinase activity. Our observation that lethal overproduction of CLB2 causes arrest in a late stage of anaphase not metaphase (see fig. 7) is consistent with this interpretation.

Our data suggest that while the broad regulatory scheme for cell cycle control in *S. cerevisiae* is similar to the one found in organisms as diverse as human, *Xenopus* and *S. pombe*, the details may differ substantially; fig. 8 summarizes the evidence for and against the model shown in fig. 5.

We are currently exploring the role of *CDC15* gene product in the destruction of CDC28/CLB2 kinase. Investigations of the putative regulatory pathways which may intersect with the control of the phosphorylation state of CDC28 are also underway.

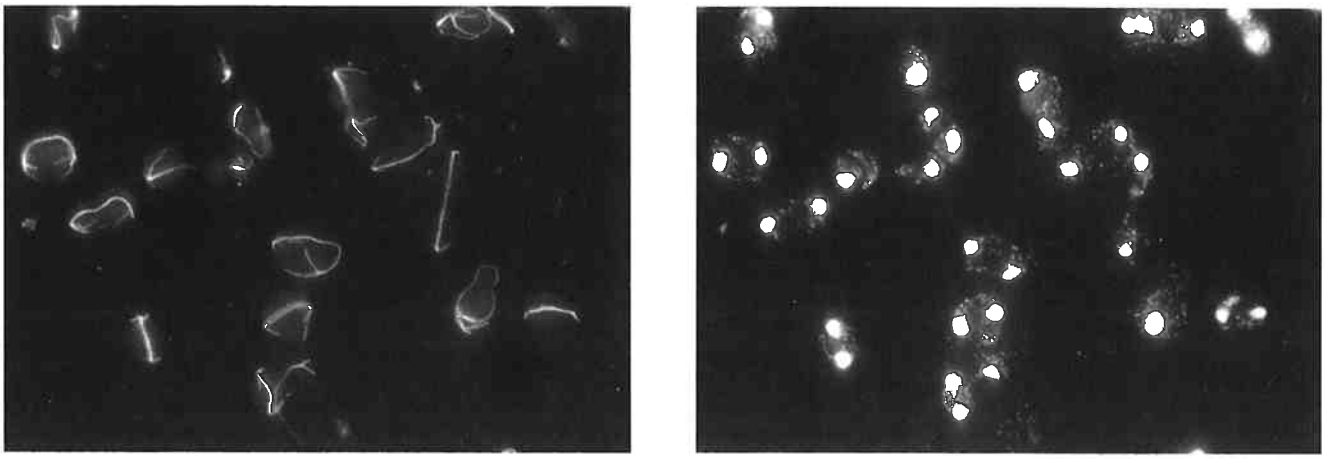


Figure 7

FOR

1) CLB´s are required for the formation of a mitotic spindle.
2) CDC28 kinase is associated with CLB2
3) CDC28 Y19 phosphorylation is cell cycle regulated.

e.g.	G1	S	G2	M	A
	-	+	+	-	-

AGAINST

1) Y19 → F19 mutation NO phenotype
2) CLB2 / CDC28 kinase is high in G2.
3) M → A can take place without CLB / CDC28 kinase destruction and overproduction of CLB2 leads to an anaphase not a metaphase arrest.

Figure 8.

A new role for MCM1: cell cycle regulation of SWI5 transcription

David Lydall

As cells of the budding yeast *Saccharomyces cerevisiae* proceed through the cell cycle they transiently activate the transcription of several genes. For example, in late G1 at START when cells become committed to enter S phase, many of the genes involved in DNA replication become transcriptionally activated. However, other genes respond to different types of cell cycle control. *SWI5*, which encodes a transcription factor for the *HO* endonuclease gene, and *CLB1* and *CLB2* , which encode B type cyclins, are only transcribed at later stages of the cell cycle during S, G2 and M phases.

SWI5 is required for mating type switching in *S. cerevisiae*. Mating type switching is induced when the HO endonuclease introduces a double strand break to the *MAT* locus of a haploid yeast cell. The process is tightly regulated due to control over *HO* gene transcription and *SWI5* is just one of several genetically identified regulators of *HO* transcription. *S. cerevisiae* cells divide asymmetrically to produce a mother cell which transcribes *HO* and switches, and a smaller daughter cell which does not transcribe *HO* and which therefore does not switch. Mother cells only transcribe *HO* in a brief post-START window of their cell cycle i. e. after they are committed to cell division but before they have replicated their DNA and thus *HO* is one of the large class of yeast genes which are only transcribed in late G1 as cells become committed to enter S phase. One of the reasons why mother but not daughter cells express *HO* is that *SWI5* gene expression is cell cycle regulated. *SWI5* RNA begins to accumulate at some point appreciably later than START, probably in middle or late S phase, and declines as cells complete mitosis and enter G1. If *SWI5* is expressed ectopically during G1 then daughter cells are able to switch mating type.

We have identified a 55bp promoter sequence that appears to be responsible for restricting *SWI5* transcription to the late S, G2 and M phases of the cell cycle. Two proteins, MCM1, a previously described transcription factor, and SFF (**S**WI **F**ive **F**actor, a newly identified factor) bind this sequence *in vitro*. MCM1 binds the DNA tightly on its own but SFF will only bind as part of a ternary complex with MCM1. We observe a strong correlation between the ability of mutated *SWI5* promoter sequences to form a ternary MCM1-SFF containing complex *in vitro* and to activate transcription *in vivo* which suggests that efficient transcription requires that both proteins bind DNA. Through its interactions with cell type specific co-activators and co-repressors MCM1 controls cell type specific expression of pheromone and receptor genes. By analogy we propose that it is SFF that enables MCM1 to function as a part of a cell cycle regulated transcription complex.

PUBLICATIONS SUBMITTED 1991

1. *Nasmyth, K., and Dirick, L. (1991): The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast. Cell* ***66****, 995–1013.*
2. *Dirick, L., and Nasmyth, K. (1991): Positive feedback in the activitation of G1 cyclins in yeast. Nature* ***351****, 754–757.*
3. *Moll, T., Tebb, G., Surana, T., Robitsch, H., and Nasmyth, K. (1991): The role of phosphorylation and the CDC28 protein kinase in cell cycle regulated nuclear import of the Saccharomyces cerevisiae transcription factor SWI15. Cell* ***66****, 763–758.*
4. *Nasmyth, K. (1991): Cell cycle research proliferates. The New Biologist* ***3****, 955–958.*
5. *Lydall, D., Ammerer, G., and Nasmyth, K. (1991): A new Role for MCM1 in yeast: Cell Cycle Regulation of SWI5 Transcription. Genes and Dev., in press.*
6. *Nasmyth, K., Dirick, L., Surana, U., Amon, A., and Cvrckova, F. (1991): Some facts and thoughts on cell cycle control in yeast. Spring Harbor Symposium, in press.*
7. *Amon, A., Surana, U., Muroff, I., and Nasmyth, K. (1991): Regulation of p34Cdc28 tyrosine phosphorylation is not required for entry into mitosis in S. cerevisiae. Nature, in press.*
8. *Gartner, A., Nasmyth, K. A., and Ammerer, G. (1991): Signal transduction in S. cerevisiae requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Submitted.*
9. *Patel, G., Nasmyth, K., and Jones, N. (1991): A novel method for the isolation of recombinant-Baculovirus. NAR, in press.*

Gene Function in Mammalian Development and Differentiation

Erwin WAGNER	Senior scientist
Thomas von RÜDEN	Staff scientist
Adriano AGUZZI	IMP Postdoc
Frank HILBERG	IMP Postdoc
Zhao-Qi WANG	IMP Postdoc
Gabor VERES	IMP Postdoc (until June 1991)
Warren ALEXANDER	MRC Postdoc
Agi GRIGORIADIS	MRC Postdoc
Marion FUNG	FFWF Postdoc(since April 1991)
Uta MÖHLE-STEINLEIN	IMP Technician
Stefanie KANDELS	IMP Technician (until May 1991)
Ingrid ANHAUSER	IMP Technician (since February 1991)
Laura STINGL	IMP Technician (since September 1991)
Erika SCHELLANDER	FFWF Technician (until June 1991)
Jun LIANG	FFWF Technician (since July 1991)
Ulrike BURKERT	IMP Grad. student
Friedemann KIEFER	IMP Grad. student

Our research programme continues to focus on the function of genes in stem cell differentiation, development and disease. We are specifically interested in analyzing the function of growth control genes, in studying the molecular events leading to tumour formation and in generating mouse models for human genetic diseases. For these studies we are using: (i) Transgenic mice produced by introducing recombinant genes into the germ line by microinjection of DNA into fertilized eggs. (ii) Embryonic stem (ES) cells as target cells for gene transfer to ectopically express foreign genes as well as to inactivate gene function by homologous recombination. Genetically altered ES cells are introduced into mouse embryos in order to analyze the consequences of the altered genotype in chimeras and their transgenic offspring. (iii) Retroviral gene transfer to express growth factor receptor genes in murine bone marrow cells *in vitro* and *in vivo* to study their function during hematopoietic development.

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

A novel target cell for c-fos -induced oncogenesis: Development of chondrogenic tumours in ES cell chimaeras

Zhao-Qi Wang, Agi Grigoriadis and Uta Möhle-Steinlein

Embryonic stem (ES) cells were used to investigate the target cell specificity and consequences of ectopic c-*fos* expression during embryonic development. We have previously shown that chimaeric mice generated with different ES cell clones selected for high exogenous c-*fos* expression were not affected during embryonic development, however, a high frequency of cartilage tumours developed as early as 3–4 weeks of age. Expression of exo-genous c-*fos* RNA and Fos protein was observed during development but was highest in tumour tissues, predominantly in differentiating chondrocytes (Wang et al., 1991).

Clonal tumour-derived cell lines were established which express high levels of c-*fos* as well as the cartilage-specific gene type II collagen. All cell lines give rise to cartilage tumours *in vivo*, some of which also contain bone. These data suggest that chondrogenic cells and earlier progenitors are specifically transformed by Fos

and therefore represent a novel mesenchymal target cell for c-*fos* overexpression. Interestingly, high levels of exogenous c-*fos* did not interfere with endogenous c-*fos* expression suggesting that trans-repression of the endogenous c-*fos* gene does not occur in the chondrogenic cells. Analysis of other AP-1 genes showed that c-*jun* and *fra*-1 were co-expressed with exogenous c-*fos* in the chondrogenic cell lines. Thus, these data suggest that selective transcriptional activation of some AP-1 genes may be responsible for cartilage tumour formation in the *fos* chimaeras.

We are currently investigating whether c-*jun* and *fra*-1 are cooperating genes in cartilage tumour development, and which target genes are responsible for c-Fos action. More importantly, generating mouse mutants with inactivated c-*fos* would offer an opportunity for dissecting the biological functions of c-Fos.

Expression of AP-1 and osteoblast-associated genes in cell lines from c-fos transgenic osteosarcomas

Agi Grigoriadis, Zheo-Qi Wang and Karl Schellander

We have previously generated transgenic mice expressing c-*fos* under the control of the murine H2-K^b class I MHC promoter which develop osteosarcomas with 100% penetrance. To investigate the potential target cells and genes which may be affected by Fos we have analyzed by *in situ* hybridization and Northern analyses the expression patterns of AP-1- and bone-associated genes in developing bones, tumours and tumour-derived cell lines.

The onset of exogenous c-*fos* expression in transgenic bones occurs between 2–3 weeks of age. *In situ* hybridization for exogenous c-*fos* at this stage demonstrated that the transgene was expressed at high levels in osteoblasts (OBs) and osteocytes and to a lesser extent in pre-OBs. Interestingly, some cells within the chondrogenic lineage also expressed c-*fos*. Thus, these results suggest that while both bone and cartilage cells are targets for ectopic c-*fos* expression in transgenic mice, only OBs are affected and give rise to osteogenic tumours.

Eleven cell lines isolated from these osteosarcomas express high levels of exogenous c-*fos* with little or no endogenous c-*fos*. 8/9 cell lines tested were tumorigenic in nude mice and the tumours are currently being characterized. Analysis of AP-1-associated genes showed that while *jun* genes were expressed at moderate to high levels, only *fra*-1 levels appeared to be correlated with levels of exogenous c-*fos*. OB marker genes were expressed at various levels, however, in contrast to tumor tissues, expression of osteocalcin was not detectable in cell lines expressing high exogenous c-*fos*. These data suggest that specific AP-1 genes may be involved in c-*fos*-induced bone tumour formation and that some OB-associated genes may be targets for Fos action. A model for the different specificity of c-Fos in mesenchymal cells in transgenic versus *fos* ES cell chimaeras is shown in Fig. 1.

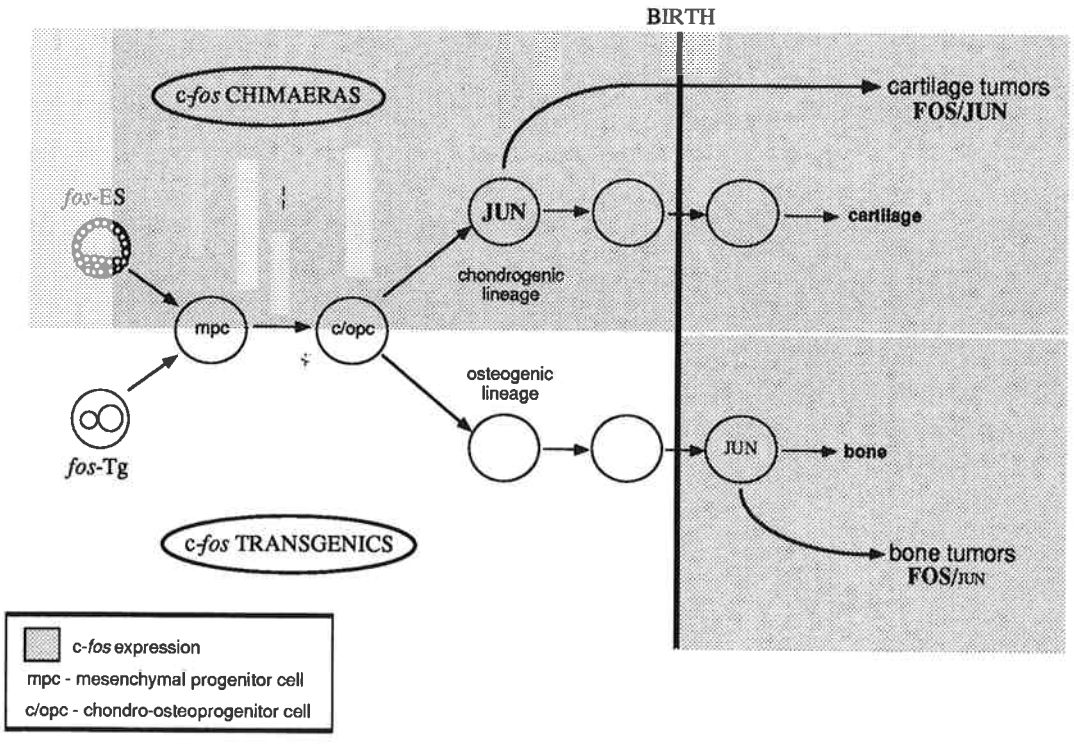


Figure 1. *A model for explaining the different phenotypes observed between c-fos chimaeras (cartilage tumours) and c-fos transgenic mice (bone tumours).*

II. The function of c-*jun* in ES cells and transgenic mice

Frank Hilberg and Karl Schellander

To investigate the role of c-*jun* during early mouse development we are generating mouse strains with either ectopic expression of the c-*jun* gene (gain of function) and/or with inactivated c-*jun* genes (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 genes under the control of strong constitutively expressed enhancer/promoter sequences. Several transgenic lines expressing the exogenous c-*jun* gene were established, which did not exhibit any obvious phenotype.

Introduction of *jun* constructs into ES cells by electroporation led to the isolation of high expressing ES cell clones. High *jun* expression did neither alter the growth parameters nor did it change the undifferentiated ES cell morphology. These ES clones were used for blastocyst injection and the generation of chimaeric mice. No significant ES cell contribution was detected in the adult chimaeras but a high rate of fetal resorptions was observed, suggesting a possible interference of c-*jun* overexpression with normal development. Histological analysis of the chimaeric fetuses at different time points during development are presently being performed.

In an attempt to inactivate the c-*jun* gene through homologous recombination we have used a promoterless *jun/neo* fusion construct to successfully inactivate one c-*jun* allele in various ES cell lines. Consecutive inactivation of the second c-*jun* allele with a promoterless *jun/hygro* fusion construct was also achieved (Fig. 2). These ES cells lack functional *c-jun* protein and do not exhibit any altered growth and differentiation properties *in vitro*. Furthermore, TPA induction of genes such as *c-fos* and *jun*B, which are thought to be regulated by c-Jun as well as transactivation of AP-1 responsive reporter constructs are not affected in these cells. The potential to differentiate *in vivo* is currently under investigation and we are also testing several chimaeras for germline transmission to obtain c-*jun* deficient mice.

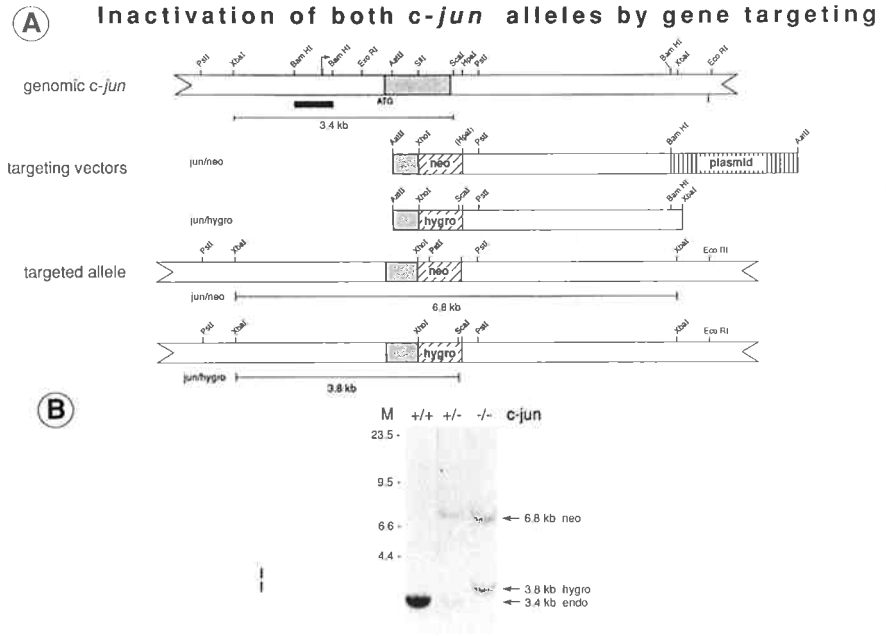


Figure 2. *Strategy and DNA analysis of single (+/–) and double (–/–) inactivated c-jun alleles in ES cells.*

III. Transgenic mouse models for human neurological disorders

Adriano Aguzzi and Ingrid Anhauser

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 underly many diseases.

Human foamy virus: an underestimated neuropathogen?

Human foamy virus (HFV) is a recently characterized retrovirus which was isolated from patients with various neoplastic and degenerative diseases. Little is known about the prevalence of HFV in the population, and it has not yet been possible to establish causal links between HFV and any human disease. Like HTLV and HIV, HFV encodes the structural retroviral genes and an additional region containing three open reading frames, *bel*-1 to *bel*-3. It was shown that *bel*-1 encodes a transactivating protein.

We have analyzed in close collaboration with the groups of Ivan Horak and Axel Rethwilm in Würzburg, Germany, transgenic mice expressing parts of the HFV genome. We found that the HFV transgene is transiently transcribed between midgestation and birth at moderate levels in various tissues. Expression is turned off around birth, and the transgene remains dormant for several weeks. After the 5th week of age, expression resumes in a restricted range of tissues (Aguzzi et al., 1992). This second wave of expression leads to extensive accumulation of HFV transcripts in single cells, mainly in the CNS and striated muscle, and is associated with a progressive degenerative disease of these tissues (Bothe et al., 1991, Fig. 3a). These findings raise the question as to whether natural infection with HFV may also lead to similar pathologies and prompt a critical re-evaluation of its possible role as a human pathogen, particularly in neurological diseases. In addition, the HFV transgenic mouse model may prove useful for studying the molecular basis of the HFV-associated neurotoxicity, the role of individual disease-associated HFV genes and the regulation of retroviral latency.

The primary research goal will be the unequivocal identification of neurotoxic genes transduced by HFV. This aim will be pursued by i) the generation of transgenic mice carrying mutated HFV constructs and ii) by intrathecal administration of recombinant HFV proteins to wild type and HFV transgenic mice. In addition, HFV transgenes carrying deletions in the ancillary *bel* genes will be introduced into mice, and their expression pattern during development and in adult mice will be studied.

In order to follow up the accumulation of HFV gene products in target organs, we have raised rabbit antisera to bacterially expressed portions of the *gag* and *env* genes. These antisera recognize formaldehyde and paraffin stable epitopes (Fig. 3b) and can therefore be used for retrospective surveys on human neuropathological material.

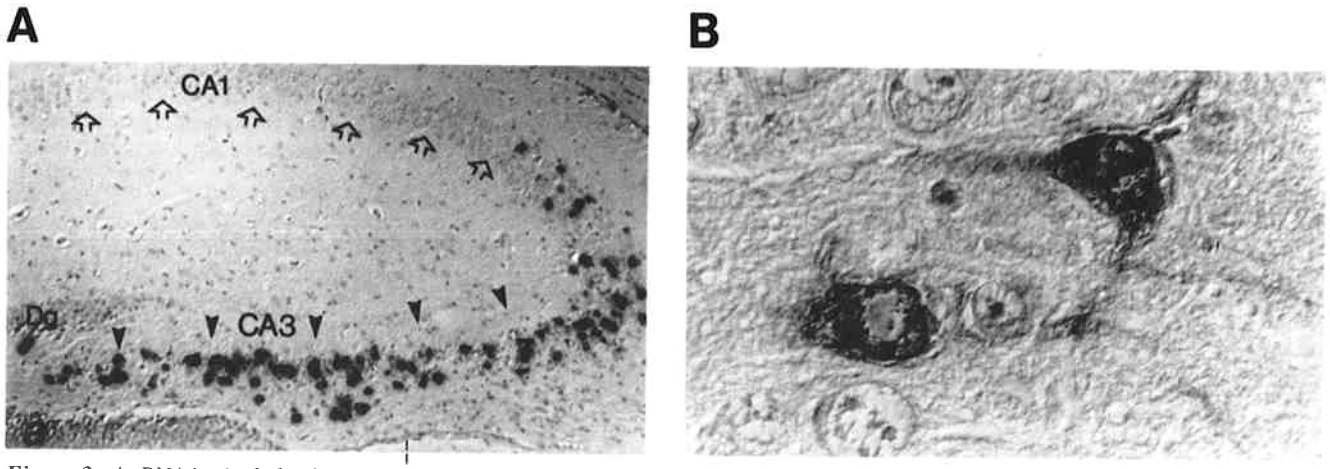


Figure 3. ***A****: RNA in situ hybridization of the hippocampus of a young HFV transgenic mouse. Many neurons in the CA3 sublayer express the transgene.*
B*: A fraction of cortical neurons in a HFV transgenic mouse reacts strongly with a gag antiserum. Note that immunoreactive material extends into axons and dendritic processes.*

Studies with the neu oncogene

The *neu* (c-*erb*B-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 one approach, we have set out to target expression of *neu* to the mouse myelination apparatus. Transgenic mice have been generated in which the activated *neu* oncogene was linked to a fragment of the myelin basic protein promoter. However, expression analysis of these mice showed that efficient expression of *neu* in the central and peripheral nervous system was not achieved, and a neoplastic or myelinopathic phenotype was not observed. Therefore, as an alternative approach we have constructed two replication-defective retroviral vectors allowing expression of the *neu* cDNA from either the retroviral long terminal repeat (*LneuT*) or from an internal thymidine kinase promoter (*NTKneuT*). The *LneuT* vector was shown to efficiently express *neu* and is currently being used for injection of midgestation mouse embryos. These experiments will allow us to analyze the consequences of *neu* expression in a broad spectrum of tissues.

IV. Molecular analysis and the role of proteolysis in the formation of endothelial tumors

Friedemann Kiefer, Agi Aguzzi and Uta Möhle-Steinlein

The middle T antigen of polyomavirus (mT) rapidly transforms endothelial cells *in vivo*, leading to the formation of endothelial tumours called heman-giomas. We have shown that mT-transformed endothelial cells (End cells) upre-gulate expression of urokinase plasmino-gen activator (uPA) and uPA receptor (uPA-R) while plasminogen activator inhibitor (PAI-1) expression is drastically reduced. End cells form morphologically aberrant cyst-like structures in fibrin gels which by addition of protease inhibitors can be corrected into tubular structures like those formed by normal endothelial cells.

In order to assess the importance of deregulated proteolytic activity for hemangioma formation *in vivo*, we have co-injected protease inhibitors and End cells into mice, which lead to a reduction of hemangioma formation. We currently pursue several other experimental approaches in order to better define the causal role of uPA/PAI-1 activity for this process: (i) overexpression of uPA in primary endothelial cells via a retroviral expression-vector, (ii) overexpression of PAI-1 in End cells and (iii) expression analysis of uPA, uPA-R and PAI-1 in hemangiomas by RNA *in situ* hybridization.

A recently characterized polyomavirus of hamster origin does not associate with pp60$^{c\text{-}src}$, but rather with another tyrosine kinase, p59^{fyn}. Using a retrovirus carrying the hamster mT we also observed hemangioma formation after infection of newborn mice, suggesting that activation of pp60$^{c\text{-}src}$ is not required for this pathology. Furthermore, we recently found that hemangiomas can also be induced in c-*src* deficient mice. This work is done in collaboration with W. Risau's group in Munich and S. Courtneidge (EMBL), R. Montesano (Geneva) and P. Soriano (Houston).

V. Early fetal hematopoiesis from *in vitro* differentiated ES cells

Ulrike Burkert and Thomas von Rüden

The potential of ES cells to differentiate into hematopoietic cells *in vitro* was investigated. When cultured in semi-solid medium four of seven ES cell lines (E14, MBL-5, CCE and D3) efficiently generated embryoid bodies (EBs) containing red blood islands. We have studied the effects of various growth factors on these early stages of hematopoietic differentiation, demonstrating that only erythropoietin modulates development of the first erythroid progenitors. In EB's derived from D3 and CCE ES cells hematopoietic cells from all six myeloid lineages were reproducibly identified. Dissociated EBs were analysed for the presence of progenitors using the *in vitro* colony forming cell (CFC) assay. All colony forming progenitors normally found in the hematopoietic organs of the developing mouse, including the „mixed" colony type, were readily detected. Detailed temporal studies showed that the various progenitors developed repro-ducibly at specific time points during ES cell differentiation. Moreover, we have tested the *in vivo* capacities of EB-derived progenitors. Despite a high frequency of CFC-Mix *in vitro*, CFU-S activity of such precursors was not observed (Burkert et al., 1991). We conclude that hematopoietic differentiation of ES cells under these *in vitro* conditions reflects complete blood cell formation in the yolk sac of the early fetus which has not progressed to the fetal liver stage. Therefore *in vitro* differentiation of ES cells provides a powerful system to study the earliest events of hematopoietic development under defined conditions.

To investigate the effect of oncogene expression on early murine hematopoiesis, ES cells were infected with a retrovirus expressing the v-*erb*B oncogene. We are currently analyzing the effects of ectopic v-*erb*B expression on ES cell differentiation *in vitro* and *in vivo*.

VI. Growth factor receptor studies in ES cells and hematopoietic cells

Marion Fung, Thomas von Rüden in collaboration with Axel Ullrich (Munich)

Growth factor receptors and oncogenes play a critical role in the regulation of normal cell proliferation and differentiation, and in cellular deregulation during tumourigenesis. To understand the function of growth factor receptors during development both *in vitro* and *in vivo*, we are attempting to ectopically express normal and mutant receptor proteins in ES cells and in transgenic mice. Specifically, the chimaeric receptors HER*erb*B and HER *kit* (EKR) were chosen for these studies. In these chimaeric molecules, the highly transforming capacity of v-*erb*B and the intracellular signal transducing domain of the developmental growth factor receptor c-Kit are controlled by the extracellular EGF receptor ligand-binding domain. Three classes of vectors were analyzed for their capacity to allow expression in ES cells: an inducible metallothionine promoter (MT), a constitutive promoter (PGK-1), and a LTR-based retroviral vector (LEN). Although all vector constructs conferred high level receptor expression in fibroblastic cells, no cell surface protein expression was detected in ES cells. Whether this ES cell-specific block in receptor protein expression is transcriptional or translational is currently being investigated.

To further study the role of growth factor receptors in hematopoietic development, several growth factor receptors including GM-CSFR, CSF-R, EGFR and the chimaeric receptors HER*erb*B and HER *kit* (EKR) were introduced into hematopoietic cells (FDC-PI) by gene transfer. In all cases, we found that selection in the presence of ligand was a prerequisite both for efficient ectopic receptor expression as well as for proliferative signalling in these cells. Furthermore, long-term high-level receptor expression appears to be regulated in a ligand-dependent manner. In the absence of ligand, cell surface receptor expression declines to minimal levels within 10 days in mixed cell populations and within 4 weeks in clonal cultures. Whether this phenomenon is restricted to hematopoietic cells is not clear. Highly permissive cells such as fibroblasts readily express functional ectopic receptors in the absence of ligand, whereas the presence of ligand does not appear to induce receptor expression in ES cells.

In a different experimental approach we are trying to interfere with growth factor receptor expression through dominant-negative mutations using the EGF-receptor as a model system. Various mutant EGF-R molecules were first tested for their inhibitory function in tissue culture cells (Redemann et al., 1992) and are presently being introduced into mid-gestation embryos as well as into ES cells and preimplantation embryos.

VII. The c-Kit receptor in mouse development and the *W* mutant phenotype

Warren Alexander

Mutations at the mouse *W* locus induce pigmentation defects, sterility and abnormal hematopoiesis. *W* lesions characteristically affect primitive cells in these lineages as well as specific differentiated progeny: in hematopoiesis *W* mutants exhibit stem cell and spleen colony forming cell (CFU-S) defects in addition to an abnormal profile of committed cells most severely affecting the erythroid and mast cell lineages. The Kit tyrosine kinase receptor, a product of the *W* locus, exhibits loss-of-function mutations in a range of independent *W* mutants suggesting a critical role in mouse development and the generation of this mutant phenotype (Wagner and Alexander, 1991).

We have established a retrovirus-based gene transfer system for the normal murine c-*kit* gene. The efficiency of c-*kit* expression in retroviral vectors was significantly greater from the retroviral 5' LTR than the internal thymidine kinase or cytomegalovirus early region promoters. Ectopic expression of c-*kit* transduced a functional growth stimulus in cells which normally lack this receptor: DA-1 myeloid cells acquired the capacity to respond to Steel factor (the Kit ligand) and fibroblasts were transformed in a ligand-dependent manner. The relationship between functional c-*kit* expression and the *W* phenotype was examined by direct gene transfer of normal Kit receptors into *W* mutant cells. In primary mast cells from mice of both the viable W/W^v and perinatal lethal *W/W* genotypes, exogenous c-*kit* expression was sufficient to rescue the response to Steel factor *in vitro*. Moreover, the capacity of W/W^v mast cells to survive and differentiate *in vivo* was also recovered. This result directly demonstrates the causal role of c-*kit* mutation in the *W* mutant mast cell deficiency (Alexander et al., 1991). We are currently investigating whether this conclusion extends to more primitive hematopoietic cells and to cells of the other affected lineages via *kit* virus infection of developing embryos or explanted hematopoietic tissue. Preliminary results suggest that the CFU-S capacity of *W* mutant bone marrow or fetal liver can also be recovered through c-*kit* gene transfer.

We are further studying the role of c-*kit* by attempting to constitutively express the receptor during mouse development. We have introduced several expression vectors into ES cells as a means to generate mice expressing c-*kit* exogenously. However, constructs driven by the herpes simplex thymidine kinase, chicken ß-actin, human metallothionine or cytomegalovirus early region promoters failed to express, despite functioning efficiently in fibroblasts. Presently we are investigating several new c-*kit* vectors in ES cells in addition to producing transgenic animals through conventional DNA microinjection or infection of mid-gestation embryos with *kit* retroviruses.

VIII. Ectopic expression of normal and mutant human EGF receptors during mouse hematopoietic development

Thomas von Rüden and Laura Stingl

We have reported earlier the expression of the normal human EGF receptor (HERc), the oncogenic viral homologue v-*erb*B and a chimaeric EGF-R/v-*erb*B (HER*erb*B) receptor on murine bone marrow cells (BMC) utilizing retroviral gene transfer. All three receptor molecules act synergistically with IL-3 in stimulating the proliferation of primary mast cells. However, only the mutant receptors, HER*erb*B and v-*erb*B, were able to maintain cell proliferation also in the absence of IL-3. As expected, the mitogenic signals of wild-type and chimaeric receptors were ligand-dependent, while v-*erb*B acts in a ligand-independent fashion. However, quite unexpected was our recent observation that not only the mitogenic effects of HERc and HER*erb*B, but also their efficient ectopic expression was dependent on the presence of EGF or TGF*a*.

In order to study the biological consequences of ectopic EGF-R expression on hematopoietic development *in vivo*, we have reconstituted mice first with BMC expressing HERc. No effects on hematopoietic development were observed, however, we were not able to demonstrate HERc expression in long-term reconstituted mice despite the presence of intact proviral DNA. Currently we believe that this might be due to insufficient serum levels of EGF or TGF*a*, allowing efficient cell surface expression.

To circumvent this problem, we expressed the ligand-independent v-*erb*B protein in reconstituted mice. All mice (10/10) developed severe myeloproliferative disorders within eight to twelve weeks after transplantation. They suffered from a systemic mast cell disease, reminiscent of *Malignant Mastocytosis* and three of them developed symptoms of an acute leukemia. Transplantation of BMC or spleen cells of four primary recipients into secondary recipients led to the development of acute leukemias, independent of whether the primary recipient already exhibited a leukemic phenotype. Leukemic cells isolated from such animals showed an undifferentiated morphology, expressed various myeloid marker antigens but also high levels of the B220 antigen, normally found on B- and pre-B-cells. However, the immunoglobulin genes were not yet rearranged, suggesting that these cells are of early myeloid origin. Genetic analysis demonstrated (i) the presence of intact as well as rearranged proviral DNA in all tissues analyzed, (ii) the clonality of the leukemias and (iii) a different clonal origin in the leukemic cells compared to the malignant mast cells. Expression of the v-*erb*B protein was confirmed by *in situ* immunohistochemical analysis.

Most recently, we have reconstituted mice with BMC co-infected with the HER*erb*B retroviral vector and a vector expressing TGF*a*. Preliminary analysis of three animals revealed that all mice had developed the symptoms of *Malignant Mastocytosis*, although the HER*erb*B protein was expressed on BMC of only two out of three mice. To test whether again acute leukemias would develop BMC were transplanted into secondary recipients and they are currently being analyzed.

This work was carried out in collaboration with Thadäus Radaszkewicz, Institute for Pathology, University Hospital, Vienna and Axel Ullrich, MPI for Biochemistry, Munich

PUBLICATIONS SUBMITTED 1991

1. *Wang, Z-Q., Grigoriadis, A., Möhle-Steinlein, U., and Wagner, E. F. (1991): A novel target cell for c-fos induced oncogenesis: development of chondrogenic tumors in embryonic stem cell·chimaeras. EMBO J.* ***10****, 2437–2450.*
2. *Wagner, E. F. (1991): Transgenic mouse models for bone and vascular diseases and gene transfer into hematopoietic cells. In: Human Gene Transfer (eds. O. Cohen-Haguenauer, M. Boiron)* ***219****, pp 227–234.*
3. *Hiller, S., Breit, S., Wang, Z.-Q., Wagner, E. F., and Schwab, M., (1991): Localization of regulatory elements controlling human MYCN expression. Oncogene* ***6****, 969–977.*
4. *Burkert, U., von Rüden, T., and Wagner, E. F. (1991): Early fetal hematopoietic development from in vitro differentiated embryonic stem cells. The New Biologist* ***3****, 698–708.*
5. *Kiefer, F., Wagner, E. F., and Keller, G. (1991): Fractionation of mouse bone marrow by adherence separates primitive hematopoietic stem cells from in vitro colony-forming cells and CFU-S. Blood,* ***78****, 2577 – 2582.*
6. *Alexander, W., Lyman, S., and Wagner, E. F. (1991): Expression of functional c-kit receptors rescues the genetic defect of W mutant mast cells. EMBO J.* ***10****, 3683–3691.*
7. *Kennedy, M., Beug, H., Wagner, E. F., and Keller, G. (1991): Novel factor-dependent erythroblast cell lines derived from reconstituted mice expressing the v-src oncogene. Blood, in press.*
8. *Wagner, E. F., and Keller, G. (1991): The introduction of genes into mouse embryos and stem cells. In: Molecular Genetics of Differentiation, Springer Verlag, in press.*
9. *Wagner, E. F., and Alexander, W. S. (1991): Of kit and mouse and man: Current Biology* ***1****, 356 – 358.*
10. *Aguzzi, A., Bothe, K., Wagner, E. F., Rethwilm, A. and Horak, I. (1991): Human foamy virus: an underestimated neuropathogen. Brain Pathology* ***2****, 61 – 67.*
11. *Brüstle, O., Aguzzi, A., Talarico, D., Kleihues, P., and Wiestler, O. D. (1991): Angiogenic activity of the k-fgf/hst oncogene in neural transplants. Oncogene, in press.*
12. *Zimmer, A., Wang, Z.-Q., Wagner, E. F., and Gruss, P. (1991): Homologous recombination in ES cells as a means to generate mice with defined mutations. In: Mechanisms of Eukaryotic DNA Recombination (M. E. Gottesman, H. Z. Vogel eds.). Academic Press, Inc., in press.*
13. *Redemann, N., Holzmann, B., von Rüden, T., Wagner, E. F., Schlessinger, J., and Ullrich, A. (1991): Anti-oncogenic activity of signalling-defective EGF receptor mutants. Mol. Cell. Biol., in press.*
14. *Rüden von T., Kandels, St., Radaszkiewicz, Th., Ullrich, A., and Wagner, E. F. (1991): Development of a malignant mast cell disease in mice reconstituted with bone marrow cells expressing the v-erbB oncogene. Blood, in press.*
15. *Aguzzi, A., Bothe, K., Anhauser, I., Horak, I., Rethwilm, A., and Wagner, E. F. (1992): Expression of Human Foamy Virus is differentially regulated during development in transgenic mice. The New Biologist, in press.*

The Group Leaders

Studies on Cell-Type Determination and Pheromone Dependent Signal Transduction in *S. cerevisiae*

Gustav AMMERER	Group leader (University of Vienna)
Anton GARTNER	PhD-student
Elisabeth PETFALSKI	Technician
Michael PRIMIG	PhD-student
Hans WINKLER	Postdoctoral fellow

A phosphorylation cascade is involved in pheromone dependent signal transduction of S. cerevisiae

Anton Gartner, Gustav Ammerer (in collaboration with Kim Nasmyth, IMP and Beverly Errede, University of North Carolina)

Yeast mating pheromones cause a receptive cell to stop proliferation and to differentiate into a gamete. Binding of the pheromone to its receptor activates a signaling cascade via the dissociation of a trimeric G-protein. The nature of the G-protein's effector is so far unknown although several gene products have been identified that function after the G-protein. Four of them called STE7, STE11, FUS3 and KSS1 are proteins with similarity to Ser/Thr protein kinases. FUS3 and KSS1 share an overlapping function in the induction of transcriptional events and are structurally related. They belong to a family of kinases whose most prominent members include mitogen activated protein kinases of vertebrate origin (MAP kinases).

We have started genetic and biochemical approaches to study the function of the FUS3 kinase. Using *in vivo* labeling procedures we have shown that similar to MAP kinases, FUS3 and KSS1 are modified by phosphorylation at threonine and tyrosine. An increase in phosphorylated protein correlates with the activation of the signal pathway. In response to the extracellular signal, the same amino acid residues are modified in FUS3 as in MAP kinases. Substitution mutations at the two potential target amino acids resulted in a biologically inactive product indicating that both threonine and tyrosine phosphorylation is essential for signal transmission. Since catalytically inactive *fus3* products still get modified at tyrosine and threonine, we can rule out that an autocatalytic mechanism is necessary for FUS3 activation. The result implies that a kinase with tyrosine specificity must exist as a signaling component. In search of this kinase we first studied the order of *STE* gene function with respect to FUS3 phosphorylation. Our results show that FUS3 activation depends on the STE11 kinase and is perhaps interdependent with the STE7 kinase. Therefore, either of these kinases is a candidate for the FUS3 kinase kinase. Currently, we are developing *in vitro* systems to address the question how these different kinases interact. Such *in vitro* systems should also help identifying important targets modified by the activated FUS3 kinase.

Cell type specific transcription in yeast

Michael Primig, Hans Winkler, Elisabeth Petfalsky, Gustav Ammerer

In haploid yeast cells, mating specificity is determined by the expression of either **a**- or α-specific peptide pheromones and receptors. The decision which set is expressed depends on the genetic information present at the mating type locus (either MAT**a** or MATα). Thereby, only the two proteins encoded by MATα contribute to this regulatory process. α1 is a positive factor required for α-specific activation and α2 a repressor inactivating the otherwise constitutive transcription of **a**-specific genes. Both regulatory proteins function by co-operation with a cell type independent DNA binding factor called MCM1. It has been shown that cell-type specific transcription correlates with the combinatorial interactions of MCM1, α1 and α2 on their respective target promoters. MCM1 belongs to a family of DNA binding proteins (called MADS box proteins) that include serum response factor (SRF), a mammalian transactivator involved in cellular responses to growth factors. Although MCM1 is central to the cell type specific activation of both **a**- and α- specific genes, the promoter function of these genes is more complex. A second layer of transcriptional control links receptor and pheromone production to the activity of their own signal transduction pathway. A defective or absent signal pathway results in severe reduction of basal levels of pheromone and receptor gene transcription. In contrast, a pathway induced by pheromone raises the expres-

sion of pheromone and receptor genes two to five fold above the basal level. The transcription factor STE12 is essential in mediating these effects. Several observations suggest that STE12 is the nuclear recipient of the transduced signal. Overexpression of STE12 suppresses the transcriptional defects caused by mutations in all other identified signal transduction components. Phosphorylation of STE12 changes in a pheromone dependent manner. STE12 recognizes a DNA sequence called pheromone response element (PRE) which has been implicated in pheromone inducible transcription. When assayed in an *in vitro* system, STE12 binds only weakly to a single binding site. However, in cooperation with MCM1 its affinity is greatly increased. Since the interaction between MCM1 and STE12 provides a physical link between cell type control and signal transduction we became interested in identifying those regions in both proteins that are necessary for their physical interaction.

STE12 is a factor without an apparent sequence similarity to any other known protein. A deletion analysis of STE12 allowed us to assign certain functions to the different regions of the protein. We were able to map the DNA binding functions to the amino terminal domain, whereas activation functions correlate with the presence of the proline and serine rich internal part of the protein. A site essential for MCM1 interaction was found close to the C-terminus of the protein. This region of about 50 amino acids is embedded within a large negative regulatory domain, which restricts DNA binding and transcriptional activation of STE12. Extensive deletions in this repressor domain have the following effects. They make STE12 binding independent of MCM1 while increasing the basal transcription rate of pheromone inducible promoters. A small deletion, destroying only the MCM1 interaction site, just leads to low receptor and pheromone production. Thus STE12's C-terminal domain may ensure high differential expression of genes that are required only for conjugation while concurrently allowing a relatively high basal activity of receptor and pheromone synthesis.

Binding assays were also used to identify STE12 reciprocating regions in MCM1. Crude deletion analyses showed that important contact sites are located just within the so-called MCM1 core domain. This is an 80 amino acid region essential for dimerization and DNA recognition. It is also the only part of the protein that shows extensive conservation between SRF and MCM1. Construction of MCM1/SRF chimaeric molecules allowed us to narrow down the region important for MCM1-STE12 co-operativity even further. Using site directed mutagenesis we are currently trying to identify amino acids that are directly involved in the physical interaction between STE12 and MCM1.

DNA binding of regulatory proteins and transcription regulation

Hans Winkler and Michael Primig

Mating specificity and conjugation are regulated by transcriptional processes. We investigated by *in vivo* footprinting methods whether any of these regulatory phenomena are due to differential DNA binding of transcriptional activators. Three questions were addressed. The first concerned α1's function in cell specialization. In the second and third we tried to relate STE12 binding to the activity of the signal transduction pathway.

(1) The activation of α-specific genes depends on the function of the ubiquitously expressed MCM1 and the cell type specific α1 protein. Since MCM1 has been proposed to function as the main transcriptional activator two hypotheses have been advanced to explain α1 dependent transcription. The first suggests that MCM1 cannot bind to the slightly degenerate α-specific binding sites unless it forms a complex with α1 (co-operative binding model). The second hypothesis emphasizes conformational effects. Although MCM1 can bind to α-specific sites *in vitro*, this site does not induce a conformational change in MCM1 that may be necessary to convert it into an activator (conformational model). We tested the different models by looking at MCM1 binding to the *MFα1* promoter. This α-specific promoter contains two functionally independent upstream activation elements. In α cells both elements are occupied by MCM1. However, in the absence of α1 (e.g. in **a** cells), only one of the sites remains occupied by MCM1, whereas the second one does not. These results indicate that both hypotheses may be biologically relevant. Although differential binding is clearly one mode by which α-specific elements are regulated, we have to assume that α1 provides functions beyond the recruitement of MCM1.

(2) Co-operative binding of STE12 and MCM1 is important for high level transcription of **a**-specific genes. *In vitro*, the interaction of MCM1 and STE12 is prevented by dephosphorylation of STE12. *In vivo* a defective signal transduction pathway severely reduces transcription of **a**-specific genes. Since it is thought that STE12 is activated *in vivo* by a phosphorylation event elicited by the pheromone dependent signal tranduction pathway one may try to correlate both observations. Therefore, we asked whether STE12 dependent footprints are absent in strains with a defective signal pathway. We found that STE12 binding is not dependent on an active pathway. Thus, STE12's interaction with MCM1 is not a target for the peromone response. Additionally, pheromone pathway independent phosphorylation events may be important for STE12 function.

(3) Upstream activating sequences that contain multiple pheromone response elements (as in the *FUS1* promoter) increase their activity up to several hundred fold upon pheromone induction. So far, STE12 is the only transcription factor shown to recognize the PRE sequence. We tried to confirm this exclusive role of STE12 by *in vivo* footprint ing analysis. Additionally, we investigated whether phero-mone dependent changes in STE12 binding can be observed *in vivo*. Our results show that all PRE elements of the *FUS1* promoter are occupied in uninduced wild type cells. No major changes are noticeable after pheromone induction, and all relevant footprints are missing in *ste12* deletion strains. Therefore, we conclude that pheromone dependent activation mechanisms operate on the already bound transcription factor, adjusting its interaction with adaptors or components of the basic transcription machinery.

REFERENCES

Sprague, G. F., Jr. (1990): Combinatorial associations of regulatory proteins and the control of cell type in yeast. Adv. Genetics **27**, *33–62.*

Dolan, J. W., and Fields (1991): Cell-type-specific transcription in yeast. Biochem. Biophys. Acta **1088**, *155–169.*

Reed, S. I. (1991): Pheromone signaling pathways in yeast. Current Opinion in Genetics and Development **1**, *391–396.*

PUBLICATIONS SUBMITTED 1991

1. *Lydall, D., Ammerer, G., and Nasmyth, K. (1991): A new Role for MCM1 in yeast: Cell Cycle Regulation of SWI5 Transcription. Genes and Dev., in press.*
2. *Primig, M., Winkler, H., and Ammerer, G. (1991): The DNA binding and oligomerization domain of MCM1 is sufficient for its interaction with other regulatory proteins. EMBO J., in press.*
3. *Gartner, A., Nasmyth, K. A., and Ammerer, G. (1991): Signal transduction in S. cerevisiae requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Submitted.*

Regulation of protein serine/threonine kinases during early G_1

Lisa M. BALLOU Group leader
Najwa CHERRADI Postdoc
Claudia PETRITSCH PhD-student

Introduction

Addition of growth factors or expression of oncogenes in quiescent fibroblasts leads to an 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 found in fibroblasts, including S6 kinases and MAP kinases. S6 kinases were originally identified by their ability to phosphorylate the S6 protein of 40S ribosomal subunits. Phosphorylation of this protein *in vivo* is associated with an increased rate of translation that is required for progression through S phase. The major S6 kinase in fibroblasts has a M_r of 70,000. Comparison of cDNA sequences shows that this enzyme is distinct from S6 kinase II (M_r=92,000), found in *Xenopus* eggs as well as fibroblasts. Both enzymes are activated *in vivo* by phosphorylation of S/T residues and can be inactivated *in vitro* by treatment with protein phosphatases 1 or 2A. Thus, it appears that S6 kinases participate in a phosphorylation cascade with at least one S/T kinase linking receptor tyrosine kinases with S6 kinases. A candidate for this link is MAP kinase, which is rapidly activated by tyrosine and T phosphorylation following mitogenic stimulation. A MAP kinase has been shown to phosphorylate and activate S6 kinase II *in vitro*, but it has no effect on the M_r=70,000 enzyme. We would like to understand how activation of the M_r=70,000 S6 kinase contributes to the mitogenic response and are interested in identifying the cellular mechanisms that control the activity of the enzyme.

Identification of the S6 kinase activator

Lisa M. Ballou

To search for the activator of the M_r=70,000 S6 kinase, I am using an *in vitro* biochemical assay that should detect an S6 kinase kinase that phosphorylates and reactivates S6 kinase. Although my working hypothesis is that the S6 kinase activator is a kinase kinase, this assay would also detect an S6 kinase regulatory subunit that would bind to the enzyme and allow it to activate itself by autophosphorylation. Active S6 kinase purified from rat liver is dephosphorylated and inactivated with phosphatase 2A. Then the protein is incubated with ATP and cell fractions and reappearance of S6 kinase activity is assayed. In designing this assay, several assumptions have been made about the putative S6 kinase kinase. First, I assume that the enzyme will be more active in stimulated cells than in resting cells. Second, it may be activated by phosphorylation, and third, its kinetics of activation are probably faster than for the M_r=70,000 S6 kinase. Some conditions that have been tried so far are: (1) Use of whole cell homogenates or low speed supernatants from resting cells or cells treated for 1, 2.5, 5, or 10 minutes with 10% serum; (2) Reactivation in the presence of Ca^{2+}, Mn^{2+} or Mg^{2+}; and (3) Use of different cocktails of phosphatase and protease inhibitors. No reactivation was seen under any of these conditions. One reason for this may be that the S6 kinase activator in extracts is too dilute to be detected. I am now using various resins (Blue Sepharose, anion and cation exchangers, phosphocellulose, etc.) to concentrate proteins from stimulated cells and the flow-through and eluted fractions will be assayed for a kinase activator as described above. In addition, purified or immunoprecipitated kinases that are known to be activated early in G_1 will be tested.

Inhibitors of S6 kinase activation

Claudia Petritsch

An increased level of cAMP early in G_1 antagonizes the growth of fibroblasts. Resting cells treated with cAMP analogs or prostaglandin E_1 (PGE_1) show a slight increase in S6 phosphorylation, due to activation of cAMP-dependent protein kinase, but no increase in DNA synthesis. Interestingly, pretreatment of cells with certain phosphodiesterase (PDE) inhibitors such as theophylline or SQ20006 almost completely blocks the EGF-induced activation of S6 kinase without affecting MAP kinase activation. PGE_1 does not have this effect. These results suggest that the block in kinase activation is not mediated by cAMP-dependent protein kinase, but may be due to inhibition of a participant in the S6 kinase cascade by the PDE inhibitors. To further test this idea, I am measuring cAMP and cAMP-dependent protein kinase activity in cells to see if high levels always correlate with loss of S6 kinase activation. These PDE inhibitors and other related compounds may be useful tools for identifying members in the pathway leading to S6 kinase activation.

S6 kinase II in fibroblasts

Najwa Cherradi

The EGF-induced increase in S6 kinase activity measured in cell extracts exhibits biphasic kinetics, with one maximum appearing after 10 minutes of stimulation and a second after 40 minutes. It has been suggested by others that this is due to activation of two distinct S6 kinases at different times: S6 kinase II is activated early and contributes most of the activity at 10 minutes, then it is down-regulated and the M_r=70,000 S6 kinase is activated. I have developed chromatographic conditions that separate S6 kinase II and M_r=70,000 S6 kinase so that I can look at the kinetics of activation of each enzyme separately. Preliminary results show that S6 kinase II contributes a small percentage of the total S6 kinase activity at early times and that its loss after 10 minutes probably cannot explain the biphasic activation kinetics. Alternative models to explain this phenomenon, such as transient activation of phosphatase 2A between 10 and 40 minutes, will be explored.

PUBLICATION

Ballou, L. M., Luther, H., and Thomas, G. (1991): MAP2 kinase and 70k S6 kinase lie on distinct signalling pathways. Nature ***349****, 348–350.*

Molecular Basis of Mouse Developmental Mutants

Denise P. BARLOW	Group leader
Pavel KUBICKA	PhD-student
Chang-Gong LIU	Visiting student
Norbert SCHWEIFER	PhD-student
Reinhard STÖGER	PhD-student

Introduction

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

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

1. The Function of Genomic Imprinting in Development and Disease

The expression of a number of genes that act in mammalian development and in some human diseases has been shown to be dependent on their parental origin – a phenomenon known as genomic imprinting. The role of genomic imprinting in mammalian development has been clearly demonstrated by nuclear transplantation and other experiments that showed that both parental genomes were essential to complete development[2]. In contrast, the role of genomic imprinting in human disease is only recently being appreciated as diseases are subject to close genetic analysis. Imprinting has been clearly implicated in a number of human genetic syndromes most notably the Beckwith-Weideman syndrome associated with fetal overgrowth[3]. We have chosen to approach the problem of the function of genomic imprinting in development and disease by molecular cloning of *Tme* (T-associated maternal effect), a genomically-imprinted mutation in the mouse[4]. Embryos that inherit a deletion of the *Tme* locus from their mother die at day 15 of development in contrast to viable embryos that inherit the same deletion from their father. The *Tme* defect was shown by nuclear transplantation experiments to be encoded within the embryonic genome and not caused by uterine or oocyte cytoplasmic defects[5]. Our approach to cloning *Tme* involved a combination of physical mapping and cloning techniques based on a chromosome 17 variant that contained a deletion of approximately 1 Mb including the *Tme* gene. This work resulted in the identification of the insulin-like growth factor-type 2 receptor (Igf2r) as a candidate gene for *Tme* and showed that this gene is only expressed from the maternally inherited chromosome in the embryo[6] . Our present goals for this project are to understand the molecular basis of parental-specific gene expression at the Igf2r locus and to correlate lack of expression of this gene with the *Tme* mutant phenotype.

I. Isolation and mapping of the mouse Igf2r genomic locus

Reinhard Stöger, Pavel Kubicka and Denise P. Barlow

In order to understand the structure of the Igf2r locus and the possible ways in which the gene can be inactivated on the paternal chromosome we have characterized the mouse genomic locus. 170 kb from the mouse Igf2r locus have been isolated on five overlapping cosmid clones and the 5' and 3' limits of the gene have been shown to span 100 kb. Restriction enzyme analyses and sequencing have been used to identify a CpG island surrounding the 5' part of the gene and transcription starts at multiple points within the island.

II. Molecular basis of genomic imprinting at the Igf2r locus

Reinhard Stöger, Chang-Gong Liu and Denise Barlow

We are proposing a model whereby Igf2r expression is limited to the maternally-inherited chromosome because of an epigenetic modification at a sequence-specific element which could be termed an "imprinting box". The location of the putative imprinting box is not clear, such a sequence by analogy to other mammalian regulatory regions, could lie within or outside the coding region of the gene. An epigenetic modification is however assumed because of the reversible nature of imprinting, i.e., a chromosome imprinted in one sex may be passed on to an individual of the opposite sex in the next generation. To test this model we have examined the entire 170 kb including and surrounding the Igf2r locus for the presence of CpG methylation sites on the maternal or paternal chromosome. Our results, using a number of methylation sensitive enzymes, have identified two sequences showing parental-specific differences in CpG methylation. We intend to examine these sequences further using genomic sequencing to characterize the full extent of these methylation differences and then to use a functional assay involving transgenic mice to ask if these sequences can confer maternal-specific expression on linked reporter genes.

III. Identification of genes causative in the imprinting process

Denise Barlow

Genomic imprinting allows chromosomes from one parent to be distinguished from the other parent. Current models would suggest gametogenesis as the stage during which chromosomes can be marked to denote their parent of origin. Identification of the exact developmental stage when these changes occur will ultimately allow us to isolate the genes involved in parental-specific "chromosomal marking". To this end we are following changes in the patterns of CpG methylation, in the Igf2r gene, during gametogenesis. Our results suggest these changes occur before the haploid stages of gametogenesis.

IV. Imprinting of the human IGF2R locus

Reinhard Stöger

The existence of genomic imprinting in humans is suggested by genetic analysis of some human diseases such as the Beckwith-Wiedeman syndrome, but so far no imprinted genes have been identified. We are examining the human IGF2R locus for the presence of imprinting in two ways. Firstly, by searching for an exon polymorphism and then to use this to examine parental-specific expression in heterozygote individuals. And, secondly by asking if the epigenetic changes identified in the mouse genomic locus also occur in the human. To this end the 5' part of the human genomic locus has been isolated and its structure analyzed.

2. The Molecular Basis of Embryonic Implantation

Norbert Schweifer

The mouse embryo attaches and implants into the uterine wall 4.5 days after fertilization. This process of attachment and unidirectional invasive implantation is a key step for the organization of the embryo. One of the developmental mutants that map to the *t complex* region on mouse chromosome 17 has been shown to be essential for the invasive step of implantation. The mutant gene has been named t^{w73}. Embryos homozygous for t^{w73} can attach but are unable to invade and implant into the uterine wall[1]. The potential importance of a gene that is essential for invasive implantation lies in the possibility that it could elucidate other biological processes that use tissue invasion such as tumor metastases.

There are two known mutant chromosomes that contain an inactive t^{w73} gene, one is named t^{w73} after the gene, and the other is t^{Lub2} which contains a 1 Mbp deletion. Our strategy for cloning the t^{w73} gene is based on *positional cloning* using the deletion of the t^{Lub2} chromosome as a guide to the maximum limits that could contain the gene, exactly the same strategy as pursued for *Tme*. We have recently shown that plasminogen maps close to the proximal breakpoint of the t^{Lub2} deletion. Plasminogen is the precursor of plasmin, a serine protease that could be predicted to be involved in tissue invasive processes. However, we have shown by DNA analysis that the *plasminogen* locus is complete in the t^{Lub2} chromosome and lies 30 kb outside the deletion. Furthermore, analysis of mRNA levels in both mutant chromosomes t^{w73} and t^{Lub2} shows that these mutants express wild type levels of plasminogen. These results obtained from two different chromosomes carrying mutations in the t^{w73} gene suggest that plasminogen does not lie at the t^{w73} locus. This project is being continued by using YACs (yeast artificial chromosomes), that can contain genomic inserts up to 1 Mb in length, to isolate in a few clones the entire 1 Mb region defined by the t^{Lub2} deletion. In collaboration with Hans Lehrach (ICRF, London), we have isolated a YAC clone from this region and it is under analysis for the presence of novel genes. Candidate genes will be tested by analyzing expression patterns in embryos containing either of the two mutant chromosomes.

3. The Molecular Basis Of Segregation Distortion In Mammals

Attila Aszodi*, Ryoma Ohi* and Denise P. Barlow (* visiting students)

t haplotypes are a natural variant form of chromosome 17 in which the t complex region is structurally rearranged. Male mice heterozygous for the t haplotype 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 distortion of the expected Mendelian segregation ratio is regulated by a number of interacting loci, tightly linked within the t complex region of chromosome 17[1]. These loci include at least four distorter loci which can act in *cis* upon a responder locus on the same chromosome or in *trans* upon the responder locus on the homologous chromosome. An understanding of the basis of this phenomenon will clearly elucidate novel mechanisms of molecular interactions.

We have used positional cloning to identify a gene expressed only in testicular germ cells during the onset of meiosis. The gene named Tcte-2 (t complex testes expressed-2) shows tight genetic and physical linkage to one of the distorter loci – T*cd-3* (t complex distorter-3) 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. Our analysis to date suggests that Tcte-2 encodes multiple transcripts, none of which contain significant open reading frames.

PUBLICATIONS SUBMITTED 1991

1. *Cox, R. D., Weydert, A., Barlow, D., and Buckingham, M. E. (1991): Three Linked Myosin Heavy Chain Genes Clustered Within 370 kb of Each Other Show Independent Transcriptional and Post-transcriptional Regulation during Differentiation of a Mouse Muscle Cell Line. Developmental Biology* **143**, *36–43.*
2. *Barlow, D. P. (1991): Preparation, restriction and hybridization analysis of mammalian DNA for pulsed-field gel electrophoresis. In "Methods, Applications and Theories in Pulsed-Field gel Electrophorersis". Eds. M. Burmeister and L. Ulanovsky, in press.*
3. *Schweifer, N., and Barlow, D. P. (1991): The Mouse Plasminogen Locus Lies at the Proximal Breakpoint of the t^{Lub2} Deletion but is not at the t^{w73} locus. Mammalian Genome, in press.*
4. *Ärtzt, K., Barlow, D., Dove, W., Fischer-Lindahl, K., Lyon, M. F., Klein, J., and Silver, L.M. (1991): Mouse Chromosome 17. Mammalian Genome* **1**, *5280–5300.*

REFERENCES

1. *Barlow, D. P.: Cloning Developmental Mutants From The Mouse t Complex. A review in press. In "The Molecular Genetics of Differentiation". Eds. Russo, Brody, Cove, Ottolenghi and Ruvkin. Springer-Verlag, Berlin, 1992.*
2. *Surani, M. A. H., Barton, S. C., and Norris, M. L.: Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature* **308**, *548–550.*
3. *Sapienza, C.: Parental imprinting of genes. Scientific American, p. 26–32, 1990.*
4. *Johnson, D. R.: Hairpintail: A case of post-reductional gene action in the mouse egg? Genetics* **76**, *p. 795–805, 1974.*
5. *McGrath, J., and Solter, D.: Maternal T^{hp} lethality in the mouse is a nuclear, not cytoplasmic defect. Nature* **308**, *p. 550–551, 1984.*
6. *Barlow, D. P., Stöger, R., Herrmann, B. G., Saito, K., and Schweifer, N.: The mouse Insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature* **349**, *84–87, 1991.*

Regulating the Activity of AP-1 to Study its Biological Role

Martin NICKLIN Group leader
Arnold REIKERSTORFER PhD-student
Ruth HERBST PhD-student
Dietmar ZECHNER Diploma student

During cell differentiation and the cell cycle, specific extracellular signals cause irreversible alterations in cell metabolism. This in turn requires sustained changes in gene expression. One simple, currently held model for how these changes might be produced is as follows. A specific molecular signal elicits a subtly particular set of changes in the intracellular signalling machinery. This has two effects, it elicits the induction of a subset of a relatively small group of transcription factors and also determines how these factors should be modified, and thus perhaps regulates their activity. The transcription factors interact combinatorially to activate the promoters of the appropriate target genes. Expression of certain target genes may result in the cell becoming irreversibly changed, or to the expression of other transcription factors that in turn result in commitment to change. Different responses might thus be produced by the expression of certain combinations of the same set of transcription factors.

One set of transcription factors that is widely supposed to be involved both in the commitment of cells to enter the cell cycle and to differentiate is the AP-1 complex, which binds to the consensus DNA element TGA$^{C}/_{G}$TCA. AP-1 consists of two families of proteins of which the prototypes are the products of the oncogenes *fos* and *jun*. Both groups of proteins include a long alpha-helical region containing heptad repeats of leucine residues which has been dubbed the "leucine zipper", because it is through this region that the AP-1 monomers interact to form dimers. Fos and Fos-like proteins do not form homodimers, whereas Jun and Jun-like proteins do. Jun homodimers can be readily disrupted by Fos monomer, to form very stable Fos-Jun heterodimers. Thus AP-1 sites can act as the targets for a large number of different molecular species, especially when one takes into account the variety of phosphorylation sites that have been identified in these proteins.

There is abundant circumstantial evidence linking AP-1 with growth control. Both *fos* and *jun* are oncogenes, that is, they interfere with growth control when overexpressed. Almost all of the genes encoding AP-1 components are highly and transiently expressed when quiescent fibroblasts are stimulated with mitogens. More direct experiments, with the expression of *fos* anti-sense RNA or with the microinjection of antibodies directed against the components of AP-1 have also suggested that AP-1 is required in order to re-enter the cell cycle.

There is also circumstantial evidence that the strong but slow increase in AP-1 activity during the differentiation of embryonic carcinoma cells (the stem cells of teratocarcinomas) is intimately tied to the process of commitment to differentiate.

We are investigating the role of AP-1 in the growth of fibroblastic cells and in the differentiation of embryonic carcinoma cells. First and most simply, we would like to know if AP-1 is required for the activation of genes that are essential for a given biological process (which we assay by the failure of that process) and then, what those essential genes are. To do this we want to achieve a controllable "knock-out" of AP-1 activity. This we are approaching by designing and expressing non-activating proteins that are able to bind and block AP-1 sites. Expression of these synthetic genes is then placed under the control of an inducible promoter in stable cell-lines. Our second approach is based on the assumption that certain biological processes that need transcriptional control through AP-1 sites may also require specific components of the complex. In order to investigate how a given component is involved, we would need to have the activity of that component under experimental control. For this reason we are attempting to design conditional (temperature sensitive) mutations of the Fos protein, based on our understanding of its structure and interaction with molecules of the Jun family of proteins. We believe that developing a general approach will allow us to make conditional mutations in other members of the B-Zip class of proteins. Efforts are being made in a number of laboratories to delete genes that encode AP-1 member proteins. A temperature sensitive mutant gene could be used to replace the missing gene, thus rescuing the phenotype of the deletion, but in a conditional manner. Initially, however, we are testing our mutated *fos* genes in the simple biological assay of transformation.

Antagonizing AP-1 in Fibroblasts

We reported last year that a fusion between dihydrofolate reductase (DHFR) and 86 amino acids of the human Jun protein (DBZj) could block AP-1 dependent transcription of a reporter gene (from a synthetic promoter containing a multimerized AP-1 site). We also had data suggesting that the protein could not be stably expressed in fibroblasts. Recently (in collaboration with Kurt Zatloukal) we have been using beta-galactosidase expression to follow the fate of individual transiently transfected cells. The results are still preliminary, but seem to confirm our view that expression of DBZj blocks cell division. We are currently testing whether this effect can be rescued by over-expressing Fos and Jun proteins, and whether there is a specific stage in the cell cycle where the block occurs.

Clearly, further investigation of the importance of AP-1 activity in the cell cycle will require the expression of the AP-1 antagonist under control of a regulatable promoter in stable cell lines. To this end we have established rodent fibroblast lines that express the hybrid activator protein Gal/ER (from Sylvia Braselmann and Meinrad Busslinger). This protein contains the hormone regulated transcriptional activator domain of the human estrogen receptor fused to the DNA-binding domain of the yeast transcription factor Gal4. When constitutively expressed in cells, the Gal/ER protein activates gene expression from promoters that contain Gal4-binding elements (GBE) only in the presence of estrogen. We have thus constructed DBZj and control (DHFR) expression vectors containing GBE-dependent promoters and we are currently establishing stable lines from Gal/ER-expressing fibroblasts. These lines will enable us to identify specific stages in the cell-cycle where AP-1 activity might be required, and eventually to identify AP-1 regulated genes that are required in the regulation of cell growth.

Antagonizing AP-1 in Embryonic Carcinoma Cells

F9 and P19 are embryonic carcinoma lines used frequently in the laboratory as models for studying cellular differentiation. F9 differentiates only in the presence of chemical inducers. Normally one uses retinoic acid (RA). F9 then yields only cells that resemble extraembryonic endodermal cells. The advantage of F9 is the uniform nature of its differentiated products. P19, by contrast, is a pluripotent cell-line which will differentiate along a variety of lineages upon aggregation. Chemical inducers may be used to direct the path of differentiation and render it more efficient and synchronous. It is also possible to culture P19 as monolayers and to cause them to differentiate into fibroblast-like cells when treated with retinoic acid. We would like to know whether antagonizing AP-1 in embryonic carcinoma cells may block the cell cycle, inhibit differentiation or perhaps block specific pathways of differentiation. We have succeeded in establishing Gal/ER expressing derivatives of the EC line F9. One such line, IEC-I gives a 50-fold induction of a GBE-dependent reporter gene in the presence of estrogen. The DBZj-expressing derivative (IEC-I/8) shows a 20-fold induction of DBZj message on estrogen stimulation. We have been able to detect the DBZj protein in estrogen stimulated IEC-I/8 cell extracts by immunoblotting with anti-DHFR antibodies and by virtue of its affinity for an AP-1 site oligonucleotide in a gel-retardation assay. By incubating nuclear extracts with Fos protein (translated *in vitro*) and gel-retardation assay, we have shown that DBZj is present (on induction with estrogen) at levels greatly in excess of the endogenous Jun protein. We expect, therefore, that there is a sufficiently high concentration of DBZj within the induced cells to swamp the endogenous Jun concentration, thus inactivating AP-1. To verify that the level of expression of DBZj upon induction is really high enough in IEC-I/8 to repress transcription that depends on AP-1 sites, we are currently differentiating IEC-I/8 and IEC-I to analyze the expression of endogenous AP-1 dependent genes.

So far, and in contrast to our results with fibroblasts, we have observed no gross change in the rate of cell division in IEC-I/8 upon expression of DBZj, suggesting that in these cells, AP-1 has little role in the control of cell division. Similarly, we have observed no difference in the rate of differentiation, nor in the morphology of the differentiated IEC/I-8 cells on induction of DBZj expression. Our preliminary findings do not support the view that the induction of AP-1 activity is a required primary event in the reprogramming of F9 cells to differentiate along the endodermal pathway. We are also investigating the expression of the characteristic markers of differentiated F9 cells, to determine whether AP-1 is involved in the the expression of effector genes of the final differentiated phenotype.

In order to analyse whether AP-1 is involved in differentiation along pathways other than that leading to extraembryonic endoderm-like cells, we have recently begun to construct derivatives of the pluripotent EC line P19 that will express the Gal/ER inducer protein. A further advantage of P19 is that its differentiated products can be passaged and cloned, hence one could derive a variety of cell types from a given P19 line.

Rational Design of Temperature Sensitive Mutants of the Fos Protein

The alpha-helix is a unit of protein structure which is stabilized by internal hydrogen bonds within its main chain. It is known to be highly co-operative in its assembly. The leucine zipper portions of Fos and Jun are unusual in that they are long alpha-helices which for most of their length appear to be exposed to the solvent on one side. The other surface makes specific contacts with the dimerizing partner. A number of studies have indicated that the zipper helices are sufficient for stable protein-protein interaction, and for orientating the DNA-binding regions. We are currently testing the effect of making and accumulating helix-destabilizing mutations within the leucine zipper of Fos, as a means to obtain a temperature sensitive mutation. Since we are working with a relatively small number of mutations, and it is highly unlikely that all of the mutations together will yield a normal phenotype, we are able to examine individual mutants in biological assays. However, we are also establishing a system for testing *in vitro* the stability to the denaturant urea of the ternary complex of mutated Fos, Jun and DNA. Stability to urea denaturation should correlate with stability to thermal denaturation.

To test for biological activity, we are transferring mutated fos sequences to retrovirus expression vectors. Cell populations expressing the mutant Fos constructs are then compared with those expressing the wild-type protein (and to those expressing the empty retroviral vector). We are characterizing the infected cells for transformed phenotype at 34°, 37° and 40°C, by examining morphology (round versus flat) and (when promising) by their ability to grow in soft agar.

Isolation of new cell cycle regulated genes in the yeast, *Saccharomyces cerevisiae*

Tillman SCHUSTER — Group leader
Wilfried ROSSOLL — PhD-student
Branislav KOVACECH — PhD-student
Wolfgang BREITWIESER — Diploma student

The investigation of the cell cycle has led to a general concept of how a cell regulates its progression through the cell cycle. Currently, it is thought that a serine/threonine kinase, $p34^{cdc2}$, plays a central role in the regulation of mitotic events. From the work with *Saccharomyces cerevisiae* there is evidence that the gene product of *CDC28*, which is the *cdc2* homologue of fission yeast *Schizosaccharomyces pombe*, forms a complex with any one of the G1 cyclins, CLN1, CLN2, and CLN3, in the G1 stage of the cell cycle. The CDC28-cyclin complex constitutes an active kinase with a specific function in cell cycle progression. This active kinase is able to drive a cell through a point in G1 called 'Start', beyond which a cell is irreversibly committed to the next cell cycle. As a result of this event, the cyclin subunits are thought to be destroyed or at least released from the complex. Experiments in *S. pombe*, marine invertebrates and now also in *S. cerevisiae* gave evidence that in G2, before mitosis, $p34^{cdc2}$ complexes with G2 cyclins to form an active kinase of probably a different specificity, whose activity then leads to progression into M phase. Also here the degradation of the cyclin subunits of the kinase is necessary for completion of mitosis. Central to this universal model for cell cycle control are post-transcriptional and post-translational events, namely protein modification and degradation. Our group is interested in the question of whether transcriptional activation of specific, yet unknown genes in yeast may play a 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 post-translational events. The finding of such genes could lead to homologous genes in higher eucaryotes exhibiting homologous function.

Screening of an ordered phage library for new cell cycle regulated genes

To find novel cell cycle regulated genes, that play a role in the control of the yeast cell cycle our strategy was first to search for all cell cycle regulated genes in *S. cerevisiae* and then later to test each for their function. We proceeded using Northern blot analysis as the most sensitive method for identifying cell cycle regulated genes. Our approach relies upon the existence of an ordered yeast genomic library provided by Maynard Olson which encompasses 80 to 90% of the haploid yeast genome in 855 overlapping phage. In the primary screen, individual EcoRI/HindIII DNA restriction fragments that correspond to those which have been ordered into a map of yeast chromosomes by Maynard Olson, are isolated from the recombinant phage. They are used as probes in Northern blots against RNA samples out of six different cell cycle stages. Transcripts identified as being cell cycle-regulated in this screen are further analysed by a secondary screen, which consists of Northern blots of temporal staged RNAs isolated from exponentially growing synchronous cultures. It enables us to establish whether or not these transcripts exhibit genuine periodicity.

This approach has the advantage that we will detect all those genes which cannot be found by standard genetic techniques. It is known that many genes are refractory to mutagenesis, while others can be redundant in sequence or function. Additionally, we can immediately map the chromosomal location of every gene of interest. This will help to discriminate genes which have already been isolated.

Using the primary screen we examined nearly 60% of the library using more than 4500 Northern hybridizations. In this screen we detected approximately 2500 distinct transcripts so far. We have been able to analyse the data derived from 300 phage (35% of the phage library), that represent 60% of the total data collected in our screen. The analysis identified approximately 160 transcripts that potentially show cell cycle dependent regulation. We tested 40 of them in the more refined secondary screen which revealed 20 transcripts exhibiting real temporal periodicity.

We began the characterization of DNA segments, whose transcription showed characteristic cell cycle dependent fluctuation. We subcloned several individual DNA fragments and sequenced them partially. The comparison of the derived peptide sequences with databases revealed whether this DNA represents a gene or part of a gene that has already been identified. The analysis helped us to identify and exclude from further research several known genes.

New cell cycle regulated genes

In our screen we were also able to identify several unknown periodically expressed genes. Most of them exhibit expression at the late G1/S boundary. Two genes, however, showed strong expression at the stage between mitosis and early G1 (*EGT1, EGT2* – early G1 transcript), two other ones in mitosis (*MST1, MST2* – mitosis specific transcript). This is the first time that genes could be detected in *S. cerevisiae* which are expressed at these two cell cycle points. Only one of the unknown genes tested so far by disruption experiments turned out to be essential for life of the cell. This gene (*LOW1*) is located in close proximity to *MST1*. The *LOW1* mRNA is a low abundance transcript and seems to fluctuate slightly with the expression of *MST1*. Its terminal phenotype after gene disruption, however, does not suggest a specific function in cell cycle control.

EGT1 and EGT2 are genes expressed in early G1

EGT1 exhibits cell cycle regulated expression peaking in very early G1. The transcript reaches its maximum level immediately after completion of mitosis. This pattern of expression is consistent with the possibility that this gene is involved in some steps just after completion of mitosis and very early in G1. The disruption of this gene, however, showed that it is not essential for viability. *EGT1* has been sequenced. Computer searches of the available databases did not reveal any close similarity to a known gene, nor any hints for a specific function.

EGT2 shows a similar cell cycle dependent expression pattern as *EGT1*. First sequencing results suggest that these genes are not related. Further characterization of these two genes are in progress.

FZF1 encodes a new Zn-finger protein

A specific DNA fragment derived from chromosome VII identified a gene which encodes a protein with potential Zn finger forming domains. This gene is constitutively expressed at a very low level and encodes a protein containing five Zn fingers (*FZF1*), three of which show close similarity to the murine Krox20 gene. The fourth and fifth finger follow after an interruption of 60 and 66 amino acids, respectively. Its similarity with known transcription factors indicates that it may serve as a transcription factor in yeast, although *in situ* immuno fluorescence studies do not support this hypothesis. Antibodies raised from peptides that were produced in *E. coli* do not localize the *FZF1* gene product in the nucleus at any cell stage.

MST1 and MST2 are highly related genes encoding membrane spanning proteins

Expression of *MST1* starts in late S or G2 and peaks in mitosis. Gene replacement showed that it is not essential for viability. Subsequent tests using Southern blots under low stringency conditions indicated the existence of a structural analogue (*MST2*). The *MST2* gene was isolated by dot blot analysis of the ordered phage library supplied by Maynard Olson. The DNA sequences revealed that they are two previously uncharacterized genes of over 70% identity. The predicted proteins include seven hydrophobic domains each with a length sufficient to span a membrane. The high similarity the hydrophobic domains show to the light-driven proton pump bacteriorhodopsin, suggests the possibility that both genes encode membrane proteins performing ion channel functions. However, since MST1 and MST2 with their seven membrane domains seem to belong to the same class of membrane proteins as the yeast pheromone receptors, it is also possible that they exhibit receptor function. Yeast strains were constructed to have deletions that inactivated both genes. The resulting strains are viable showing that the *MST1* and *MST2* genes do not encode an essential function, at least not under normal laboratory conditions. Since homologous sequences have been identified in DNA preparations of *Klyveromyces lactis* and *S. pombe* by Southern blot analysis these genes seem to be conserved in evolution.

Localization of the MST proteins in the yeast cell may help to determine the function they have in the cell. One possibility to achieve this is to raise antibodies against peptide sequences which are not enclosed by a membrane and use them in indirect *in situ* immuno fluorescence studies. Since the hydrophilic N-termini of both proteins share 90% identity we have generated polyclonal antibodies against the N-terminus of MST1 expressed in E.coli. These antibodies crossreact with MST2 protein on western blots. *In situ* immuno fluorescence studies suggest that the *MST1* and *MST2* gene products are localized in the plasma membrane.

REFERENCES

1. *Price, C., Nasmyth, K., and Schuster, T. (1991): A general approach to the isolation of cell cycle regulated genes in budding yeast, Saccharomyces cerevisiae. J. Mol. Biol.* **218,** *543–556.*
2. *Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B., and Nasmyth, K. (1991): The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell* **65,** *145–161.*

PUBLICATIONS SUBMITTED 1991

1. *Pammer, M., Briza, P., Ellinger, A., Schuster, T., and Breitenbach, M. (1991): DIT101, a cell cycle-regulated yeast gene required for synthesis of chitin in cell walls and chitosan in spore walls. Submitted.*

Biological Chemistry

Ernst WAGNER	Group leader
Berndt OBERHAUSER	Postdoc
Christian PLANK	Diploma student
Karl MECHTLER	Laboratory technician

I. Gene Delivery via Receptor-mediated Endocytosis

Ernst Wagner, Matt Cotten, Kurt Zatloukal, Christian Plank, Helen Kirlappos, Karl Mechtler and Max L. Birnstiel

Introduction

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 (1, 2; see **Fig. 1**). Complexes of transferrin-polycation conjugate with DNA molecules are internalized by cells. Uptake and expression of genes has been shown to be based on transferrin-dependent receptor-mediated endocytosis (3, 4). In certain erythroleukemic cell lines a large proportion (up to 100%) of the cell population are found to express the transfected genes. A strong correlation between polylysine mediated DNA condensation into structures with a size of approximately 100 nm and the DNA uptake by endocytosis has been found (5).

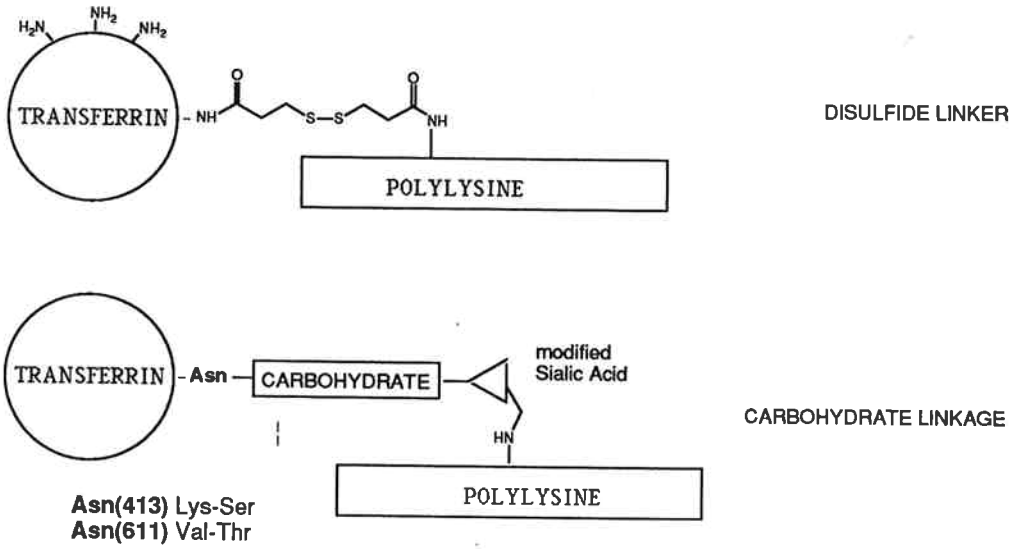


Figure 1. *Transferrin-polylysine conjugates.*

The use of synthetic and natural ligands for receptor-mediated gene delivery

The polycation conjugate approach has been extended to different receptor ligands with new targeting specificities (6). Polylysine conjugates with monoclonal antibodies directed against CD4, against CD7, or with recombinant HIV glycoprotein gp120 have been successfully used for gene delivery to T cell lines. For the purpose of hepatocyte targeting we designed a conjugate with an artificial ligand containing four galactosides on a branched carrier peptide. Using this completely synthetic, protein-free compound, gene transfer mediated through the hepatocyte-specific asialoglycoprotein receptor was demonstrated.

DNA delivered by the receptor-mediated endocytosis pathway is largely accumulated in intracellular vesicles. To overcome this limitation, we have included components that may effect endosomal or lysosomal release. DNA complexes, obtained from the combination of ligand-conjugates with a fusogenic peptide-conjugate derived from influenza virus haemagglutinin, showed considerably increased gene expression (see below). Another approach (in collaboration with David Curiel, Chapel Hill; see report group Birnstiel) exploits the capacity of adenovirus to disrupt endosomes (7, 8). In the presence of replication-defective virus transfection of hepatocytes with the galactose containing conjugate resulted in a particularly high gene transfer.

Adenovirus-polylysine DNA combination complexes as gene transfer agents

In order to ensure co-localization of the adenovirus and the DNA complex into the same endosome (see **Fig. 2**), we have constructed DNA-binding adenovirus-polylysine conjugates. A linkage between virus and polylysine was accomplished by several different strategies including direct chemical coupling, enzymatical coupling with transglutaminase, the use of an antibody bridge, or by using a streptavidine-polylysine conjugate in combination with biotinylated adenovirus. In contrast to conventional recombinant viral vectors the delivered gene is carried on the exterior of the virion, being therefore far less restricted to a particular size or sequence of the DNA (see report Stephen Phillips, group M. L. Birnstiel).

With the adenovirus-bound DNA complexes we could demonstrate gene expression at low concentrations (10 DNA molecules and 1 virus per cell), where both transferrin-polylysine alone or in combination with uncoupled adenovirus show zero expression. Combination complexes containing DNA, adenovirus-polylysine and transferrin-polylysine allow efficient gene transfer to cells that do not have appropriate receptor numbers for adenovirus. Furthermore, we were able to show efficient gene transfer to cells that are difficult to transfect, such as B-cells, to chicken bone marrow (in collaboration with Martin Zenke) as well as mouse bone marrow or to embryonal stem cells (together with Kurt Zatloukal and Matt Cotten).

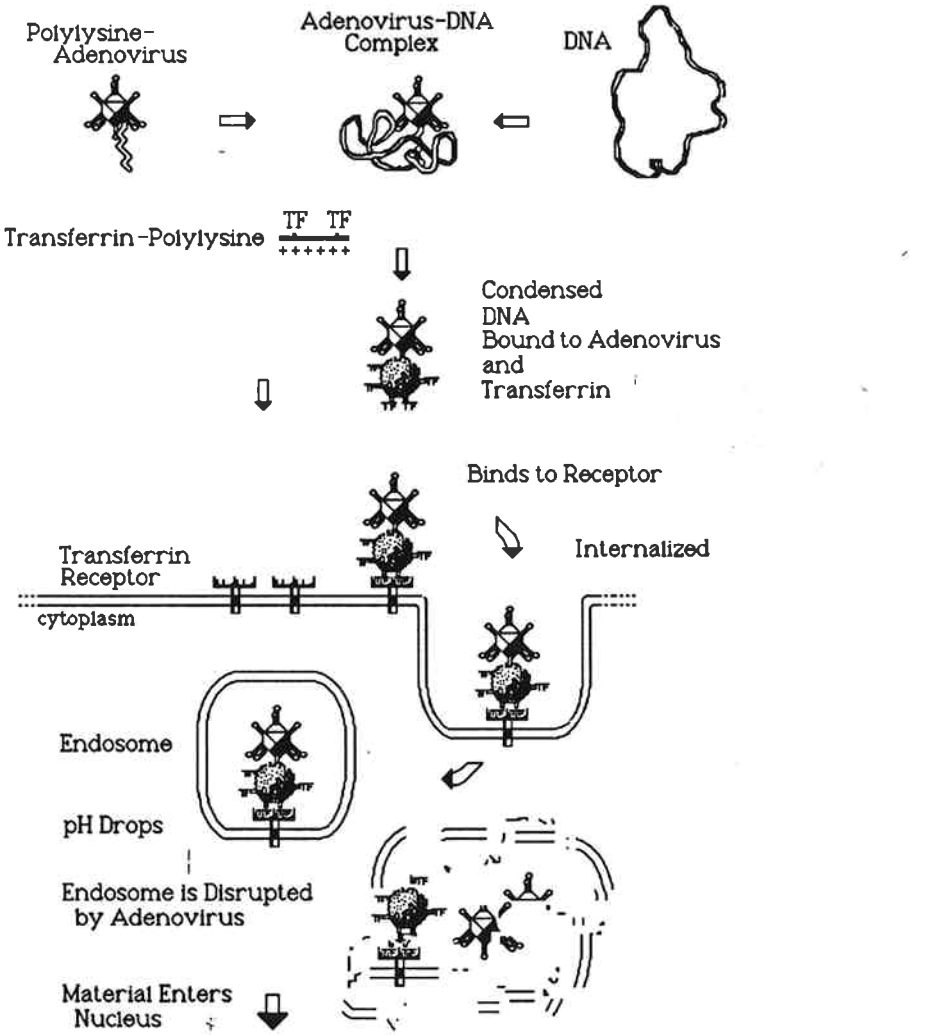


Figure 2. *Gene transfer using transferrin-adenovirus-polylysine DNA combination complexes.*

Influenza virus haemagglutinin HA2 N-terminal fusogenic peptide promotes transferrin-mediated gene delivery

An alternative approach to produce lysosomal escape utilizes the well known mechanism of influenza virus. Instead of using a whole virus only the fusogenic domain was selected as effective component in a mixed complex with DNA and transferrin-polylysine.

The viral haemagglutinin glyco-protein (HA), after the uptake of influenza virus into the cell by receptor mediated endocytosis, specifically mediates fusion of the viral membrane and the host cell endosomal membranes at the acidic pH of the endosomes. The N-terminal sequence of the HA_2-subunit is known to be responsible for this process. Peptide analogues of the 20 amino acid N-terminal sequence of HA_2 are able to fuse artificial membranes and cause leakage of aqueous liposomal contents (8). Such a peptide was synthesized by solid phase peptide synthesis and conjugated to polylysine. The fusogenic properties of the peptide and its conjugate were confirmed by a

liposome leakage-assay which measures the release of a fluorescent dye from phosphatidylcholine-liposomes upon addition of the peptide. Combination DNA complexes containing these peptide conjugates and transferrin-polylysine show considerably enhanced gene transfer efficiencies in different cell types, such as in erythroleukemic cells (K562), epithelial cells (HeLa), or hepatocytes (TIB73 cells; see **Fig. 3**).

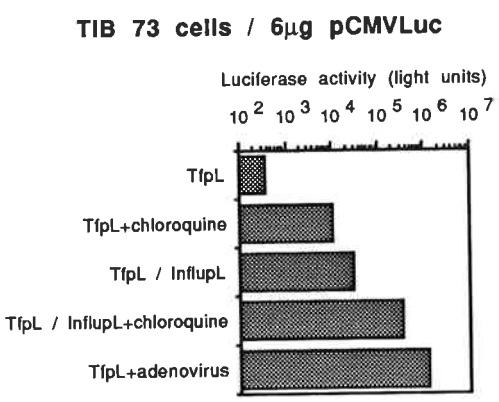


Figure 3. *The effect of chloroquine, of influenza peptide-polylysine conjugate (InflupL), the combination of both agents, or of defective adenovirus dl312 on transferrin-polylysine (TfpL) mediated gene delivery to TIB73 hepatocytes.*

REFERENCES

1. *Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L. (1990): Proç. Natl. Acad. Sci. USA* ***87****, 3410–3414.*
2. *Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991): Bioconjugate Chem.* ***2****, 226–231.*
3. *Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M. L. (1990): Proc. Natl. Acad. Sci. USA* ***87****, 3655–3659.*
4. *Cotten, M., Laengle-Rouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., and Birnstiel, M. L. (1990): Proc. Natl. Acad. Sci. USA* ***87****, 4033–4037.*
5. *Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991): Proc. Natl. Acad. Sci. USA* ***88****, 4255–4259.*
6. *Cotten, M., Wagner, E., and Birnstiel, M. L. (1991): Methods Enzymol., in press.*
7. *Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991): Proc. Natl. Acad. Sci. USA* ***88****, 8850–8854.*
8. *Wharton, S. A., Martin, S. R., Ruigrok, R. W. H., Skehel, J. J., and Wiley, D. C. (1988): J. Gen. Virol.* ***69****, 1847–1857.*

II. Antisense Oligonucleotides and RNA as Potential Tools for Gene Inhibition

Matt Cotten, Karl Mechtler, Berndt Oberhauser, Gotthold Schaffner, Walter Schmidt and Ernst Wagner

Introduction

Many diseases can be traced back to the alteration and malfunction of specific genes. Such genetic disorders may disrupt the normal cell regulation mechanism either by loss of expression of essential gene products, or by overexpression or expression of deleterious forms of mutated genes. The accumulating information on the involved genes and their nucleic acid sequences may be used for inhibition of gene expression by artificial antisense oligonucleotides that recognize and bind complementary single-stranded nucleic acids like messenger-RNA with high specificity. This purely sequence-based masking of gene expression facilitates the study of individual gene function and eventually may lead to a promising new mode of cancer therapy, and to the treatment of viral and other diseases (reviews: 1,2).

Small antisense oligonucleotides (ON's) and ribozymes may be useful tools both for the study of gene regulation and inhibition of genes. However, the inefficient uptake of the highly charged nucleic acid molecules into cells presents a substantial limitation to their application, and once imported, unmodified ON's are subjected to a rapid nuclease degradation within the cell. Therefore modifications of the ON's, with the aim of obtaining a more efficient intracellular transport and protection against hydrolysis by nucleases, but retaining a strong binding to the complementary nucleic acid targets, may increase the ONs' inhibitory potential.

Thiocholesterol-modified 2'-O-methyl-oligoribonucleotides show enhanced affinity for liposomes and cells

2'-O-Methyl-oligoribonucleotides, which have proven to be potent antisense inhibitors *in vitro* (3,4), were provided with a lipophilic anchor by linking an organic-soluble amidinium salt of a 3'-(pyridyldithio)-modified 2'-OMe-RNA to thiocholesterol in dichloromethane-methanol solution. This bioreversible linkage of a membrane-insertable cholesteryl moiety via a disulfide bond, which is likely to be cleaved in the reductive environment of the cytoplasm, should facilitate uptake by cells.

To prove this hypothesis, cultured cells were incubated with thiocholesteryl-modified 2'-OMe-RNA. The association of these modified oligonucleotides to TIB 73 liver-cells (see **Fig. 4**) was 100-fold higher as compared to the unmodified 2'-OMe-RNA in serumfree medium and about 10 to 30-fold higher in the presence of 10 % calf serum. Analog results were obtained with non-adherent HL-60 cells. During prolonged incubation release of the oligonucleotide from the thiocholesteryl-moiety through cleavage of the disulfide bond could be demonstrated (5).

Furthermore the lipophilic modification of the 2'-OMe-RNA resulted in a marked increase of incorporation-efficiency into liposomes prepared by the reverse-phase evaporation method (**Fig. 4**). As a consequence of the oligonucleotides being inserted into both sides of the the lipid-bilayer, half of the 5'-^{32}P-labelled oligonucleotides was susceptible to alkaline phosphatase which could only act on the outside of the liposomes. These new oligonucleotide-bearing liposomes may be interesting candidates for the delivery of antisense compounds into cells.

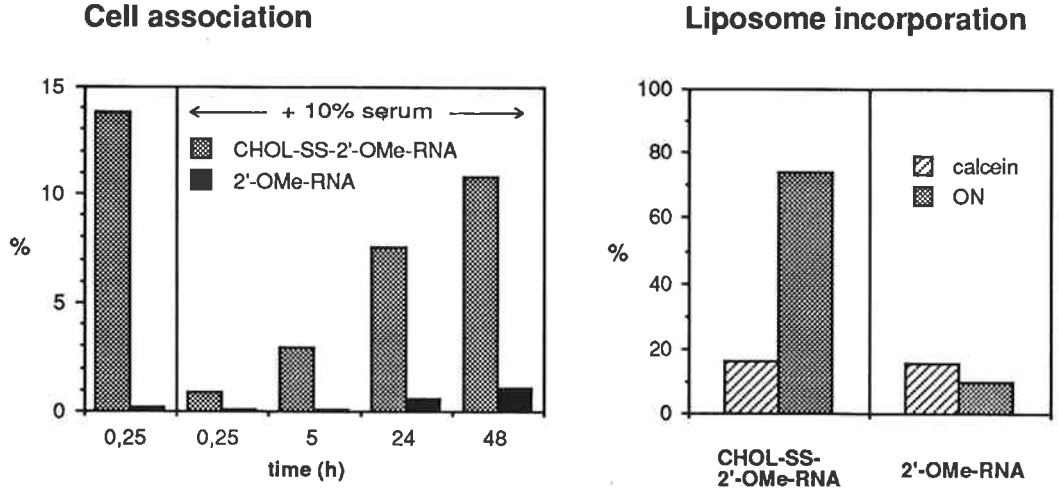


Figure 4. Special properties of 2'-O-methyloligoribonucleotides linked to thiocholesterol. Enhanced association to TIB73 cells (in the absence of serum, or with 10 %serum), and increased incorporation into liposomes.

RNA inactivation by antisense DNA and RNA in cellular biological systems

Antisense strategies can be divided into two simple categories, inhibition from without, in which *in vitro* synthesized antisense molecules are supplied to cells, or inhibition from within, which involves the introducing DNA encoding an antisense gene into the target cell.

One target system that is under investigation is the chronic myelocytic leukemia (CML). More than 90% of CML patients have structural alterations in their leucocyte DNA caused by the translocation of *c-abl* from chromosome 9 to the breakpoint cluster region (*bcr*) on chromosome 22, resulting in the formation of *bcr-abl* hybrid genes (6,7). It has been demonstrated that normal antisense ON's directed against *c-abl* can inhibit the growth of normal hematopoietic progenitor cells (7).

In order to identify the most efficient approach for inhibition a model system has been established (see report Walter Schmidt, group Birnstiel). As target for antisense inhibition a vector which carries the bcr-abl joining sequence upstream of the coding region of the luciferase gene has been stably introduced into K562 cells. After incubation with bcr-abl antisense oligonucleotides reduction of luciferase activity has been observed.

So far, the best results have been obtained with 2'-O-methyl-oligoribonucleotide-phosphorothioates. This class of modified oligonucleotides also has been found to be most effective in an anti c-myc inhibition assay with cultured HL60 cells. Furthermore, these thioate analogs that are very efficient *in vitro* and stable upon incubation with cells. But,on the other hand, with cultured cells the compounds are effective only at rather high concentrations (about 20 µmolar) that are near toxicity. Therefore, in order to reduce the effective dose, there is a need for improved internalization into the cells. As we have already a very effective gene transfer system (see above), currently investigations based on the second approach, the inhibition from within the cells, are in progress.

REFERENCES

1. *Zon, G. (1988): Pharmaceutical Research, Vol.* ***5****, 539–549.*
2. *Helene, C., and Toulme, J.-J. (1990): Biochim. Biophys. Acta* ***1049****, 99–125.*
3. *Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, C. R., Schaffner, G., Wagner, E., and Birnstiel, M. L. (1991): Nucleic Acids Res.* ***19****, 2629–2635.*
4. *Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M., and Noe, C. R.: Nucleic Acids Res., in press.*
5. *Oberhauser, B., and Wagner, E.: Nucleic Acids Res., submitted.*
6. *De Klein, A., van Kessel, A. G., Grosveld, G., Bartram, C. R., Hagemeijer, A., Bootsma, D., Spurr, N. K., Heisterkamp, N., Groffen, J., and Stephenson, J. R. (1982): Nature* ***300****, 765–767.*
7. *Caracciolo, D., Valtieri, M., Venturelli, D., Peschle, C., Gewirtz, A. M., and Calabretta, B. (1989): Science* ***245****, 1107–1110.*

PUBLICATIONS SUBMITTED 1991

1. *Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991): Transferrin-polycation DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc. Natl. Acad. Sci. USA* ***88****, 4255–4259.*
2. *Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991): DNA-Binding Transferrin Conjugates as Functional Gene Delivery Agents: Synthesis by Linkage of Polylysine or Ethidium Homodimer to the Transferrin Carbohydrate Moiety. Bioconj. Chemistry* ***2****, 226–231.*
3. *Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, Ch. R., Schaffner, G., Wagner, E., and Birnstiel, M. L. (1991): 2'-0-Methyl, 2'-0-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribo-nucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event. Nucl. Acids Res.* ***19****, 2629–2635.*
4. *Cotten, M., Wagner, E., and Birnstiel, M. L. (1991): Receptor Mediated Transport of DNA into eukaryotic cells. A chapter in Methods in Enzymology, March 1991, in press.*
5. *Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991): Adenovirus enhancement of transferrin-polylysine mediated gene delivery. Proc. Natl. Acad. Sci. USA* ***88****, 8850–8854.*
6. *Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M., and Noe, Ch. R. (1991): A simple procedure for the preparation of protected 2'0-methyl or 2'0-ethyl ribonucleoside-3'-0-phosphorami-dites. Nucl. Acids Res.* ***19****, 5965–5971.*
7. *Oberhauser, B., and Wagner, E. (1991): Effective incorporation of 2'0-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol. Nucleic Acids Res., in press.*
8. *Curiel, D. T., Agarwal, S., Romer, N., Wagner, E., Cotten, M., Birnstiel, M. L., and Boucher, R. C. (1991): Gene transfer to respiratory epithelial cells via the receptor-mediated endocytosis pathway. American Journal of Respiratory Cell and Molecular Biology, in press.*
9. *Curiel, D. T., Wagner, E., Cotten, M., Birnstiel, M. L., Li, Ch., Loechel, St., Agarwal S., and Hu, P. (1991): High efficiency gene transfer by adenovirus coupled to DNA-polylysine complexes via an antibody bridge. Human Gene Therapy, in press.*
10. *Zatloukal, K., Wagner, E., Cotten, M., Phillips, St., Plank, Ch., Steinlein, P., Curiel, D., and Birnstiel, M. L. (1991): Transferrinfection: a highly efficient way to express gene constructs in eukaryotic cells. Submitted to the Annals of the New York Academy of Sciences.*

Analysis of the Genetic Principles of Tumour Suppression

Andreas WEITH	Group leader
Paula M. STAPLETON	Postdoc
Christoph M. BARNAS	PhD-student
Christoph BRUNNER*	PhD-student
Wilfried ELLMEIER	PhD-student
Elke KLEINER	Laboratory technician

* present address: Boehringer Mannheim, Penzberg, FRG

Introduction

Recessive genetic alterations of certain genes are suggested to be causally involved in the process of tumourigenesis. Loss of gene function due to homozygous mutations which affect genes designated as tumour suppressor genes have been reported in a number of systems (for a review see Green 1988). Initial evidence for the location of such genes has often been gained by cytogenetic analyses identifying a consistently rearranged chromosomal region.

Our group is concentrating on the analysis of human chromosome 1p. Both cytogenetic and genetic linkage analyses suggest that genetic information contained in this region is involved in different cancer types, including neuroblastoma and primary hepatoma. In initial experiments, performed in the laboratory of M. Schwab (DKFZ Heidelberg), we have generated a panel of chromosome 1p-specific DNA probes by microdissection and microcloning (Martinsson et al. 1989). Using loss of heterozygosity analyse we identified a neuroblastoma-specific consensus deletion at 1p36.2–p36.1 that spans approximately 7 to 8 Megabasepairs (Mbp) of DNA (Weith et al. 1989). Subsequent investigation of six primary hepatomas (in collaboration with D. Simon and B. B. Knowles, Wistar Institute, Philadelphia) also revealed consistent allelic loss in the 1p36–p35 region, though precise borders of a consensus deletion remained undefined (Simon et al. 1991). In comparison, analyses of colorectal tumours also revealed consistent loss of alleles on 1p (Leister et al. 1991) but in a clearly more proximal region of the chromosome arm. Hence at least two loci that are involved in tumourigenesis may reside on the short arm of chromosome 1.

Further investigations of our group on the chromosome 1p region aim I) to define more precisely the borders of the consensus deletion in primary liver tumours, II) to establish a linkage map of the 1p36 region as a basis for the positional cloning of genetic elements and III) to identify and molecularly clone genes mapping to the region of our interest.

Consensus Deletion in Primary Liver Tumours

Paula M. Stapleton, Elke Kleiner and Andreas Weith *(in collaboration with D. Simon and B. B. Knowles, Wistar Inst., Philadelphia, and J. Funovics, Franz-Josef-Spital, Vienna, Austria)*

The initial hepatoma analysis was extended by collecting tissue of patients with primary liver tumours. At present DNA and RNA has been isolated from eight tumours and the corresponding normal liver tissue. In addition, tumour material was used for in vitro cell culture.

Genomic DNA's of six tumours and the corresponding normal tissue so far were used for a loss of heterozygosity analysis. Five of them displayed allele losses at 1p36 loci. Thus, a total of ten out of the twelve tumours studied so far show deletions in the 1p36 region. The high incidence of deletions in this cancer type compares with the frequency observed in neuroblastomas. We therefore suggest that genetic information involved in liver tumourigenesis maps to the distal 1p region.

One of the tumours, designated HV4T-E, could successfully be taken into culture. The tumour was diagnosed as cholangiocellular carcinoma, a carcinoma of bile duct epithelium. Cholangiocarcinomas comprise about 10% of the primary liver tumours. Cultured cells revealed a transformed phenotype since they grew both, anchorage and serum independent. When DNA was isolated from early passage cells (p. 3) and analyzed for loss of heterozygosity, allelic loss was apparent at 1p36–p35 loci. Preliminary karyotype analysis of HV4T-E cells revealed a non-balanced translocation on one of the chromosome 1 homologues with the breakpoint mapping to 1p35. The breakpoint in this tumour may delineate the proximal border of a consensus deletion in liver tumours. We therefore suggest that the region consistently affected by deletions may match in hepatomas and in neuroblastomas. More detailed analysis will discover whether the same genetic information is involved in the tumourigenesis of both cancer types.

Physical Linkage Analysis of Probes from the Chromosome 1pter–p35 Region

Christoph M. Barnas, Christoph Brunner, Wilfried Ellmeier, Elke Kleiner and Andreas Weith

The localization and molecular cloning of genes by means of positional cloning requires detailed knowledge of the ge-nomic environment of the locus and a suf-ficiently high number of markers in the re-gion of interest. We have therefore begun to establish a physical linkage map of the 1p36 region using pulsed field gel electrophoresis (PFGE). In addition to the probes already present we generated three more microclone libraries of subregions of the 1pter–p34 area, one of which was meant to cover the NB consensus deletion. At present a total of 123 single copy probes that map to 1pter–p35 have been isolated from the libraries. 53 of them were physically linked to 13 individual linkage clusters by comparing their hybridization patterns on PFGE blots of normal human DNA. The individual linkage clusters were between 900 kbp and 5.6 Mbp in size and each contained between three and nine probes. Taken together the lengths of all clusters when covered approx. 19.5 Mbp of DNA. Since we estimate the entire 1pter–p35 region to comprise less than 30 Mbp of DNA, we suggest that the linkage clusters cover more than 60% of this region. Additional experiments employing partial Not I digestion and PFGE separation of fragments up to approx. 6 Mbp are presently in progress to link the different clusters. In parallel, in-situ hybridization of probes in the different clusters on metaphase chromosomes will give information on their location relative to one another. Recently we have also included probes of the CEPH genetic linkage map (Dracopoli et al. 1991) in our PFGE analyses. The mapping of such probes with respect to our microcloned probes will provide a link between the genetic and the physical map.

Since PFGE analysis provides the possibility to visualize DNA segments in the Mbp range we have begun to analyze neuroblastoma genomes for cytogenetically invisible homozygous rearrangements in 1p36. At present representative probes of seven different linkage clusters were hybridized to PFGE filters containing different normal and tumour DNA's. Whilst the probes of six clusters did not show any rearrangement between normal and tumour DNA's, all four probes of one particular cluster displayed extensive changes of band mobilities in a few tumours. We suggest that such aberrations may pinpoint a DNA segment with a genetic element that is inactivated in both alleles. More detailed studies of this area which according to the size of the linkage cluster covers less than 900 kbp of DNA are presently in progress.

Structural and Functional Analysis of CpG Islands in the Human Chromosome 1p36 Region

Wilfried Ellmeier, Elke Kleiner and Andreas Weith

Our search for genetic elements in the chromosome 1p36 region by positional cloning involves identifying DNA segments of particular sequence composition known as CpG islands. These islands are characterized by a relatively high C+G content (60–80% as compared to 40% in bulk DNA) and high CpG dinucleotide occurrence expected from base composition. They lack methylation at the cytosine in CpG dinucleotides (Bird 1986). Characteristically they are found in the 5' regions of many genes. As a consequence of their CpG richness and lack of methylation, islands are frequently cut by rare cutting restriction enzymes. Therefore these islands can be used as markers in hybridization analyses for the presence of genes in a chromosomal region.

DNA probes derived from microdissection and microcloning of chromosome 1pter–p36.1 were analyzed by PFGE and Southern hybridization to see whether they detected rare cutter sites in their genomic vicinity. Probes detecting multiple restriction sites were used for screening a cosmid library. Subcloned probes of the isolated cosmids containing clusters of rare cutter sites were further analyzed by Northern hybridization on filters containing RNA of (1) different tumourigenic and non-tumourigenic cell lines and of (2) neuro-blastoma cell lines. Employing this approach, we have cloned four islands so far. The corresponding transcripts have been identified for all four island-associated genes. One of the genes was found to be ubiquitously transcribed in all cell lines tested, whereas the other three probes identified more tissue-restricted transcripts. All three were transcribed at detectable levels in at least one neuroblastoma line tested.

In addition the four island-specific DNA probes were analyzed for evolutionarily conserved sequences by hybridization against zoo blots. Two probes displayed conservation in all vertebrate species screened, the other two were conserved at least in all mammalian species tested. The high degree of evolutionary sequence conservation suggests an important function of the respective gene products. The isolation of the corresponding cDNAs from cDNA libraries and a more detailed analysis of the genes is in progress.

REFERENCES

Bird, A. (1986): CpG rich islands and the function of DNA methylation. Nature ***321****, 209–213.*

Dracopoli, N. C., and 26 coauthors (1991): The CEPH consortium linkage map of human chromosome 1. Genomics ***9****, 686–700.*

Green, A. R. (1988): Recessive mechanisms of malignancy. Br. J. Cancer ***58****, 115–121.*

Leister, I., Weith, A., Brüderlein, S., Cziepluch, C., Schlag, P., and Schwab, M. (1990): Human colorectal cancer: high frequency of deletions at chromosome 1p35. Cancer Res. ***50****, 7232–7235.*

Martinsson, T., Weith, A., Cziepluch, C., and Schwab, M. (1989): Chromosome 1 deletions in human neuroblastoma: generation and fine mapping of microclones from the distal 1p region. Genes, Chromosomes and Cancer ***1****, 67–78.*

Weith, A., Martinsson, T., Cziepluch, C., Brüderlein, S., Amler, L. C., Berthold, F., and Schwab, M. (1989): Neuroblastoma consensus deletion maps to chromosome 1p36.1–1p36.2. Genes, Chromosomes and Cancer ***1****, 159–166.*

PUBLICATIONS SUBMITTED 1991

1. *Zink, D., Weith, A., Martinsson, T., and Schwab, M. (1991): Analysis of chromosome band 1p36 alterations by chromosomal in situ suppression hybridization with a microclone DNA bank. Genes, Chromosomes and Cancer* ***3****, 407–410.*
2. *Yamauchi, M., Yamauchi, N., Phear, G., Spurr, N. K., Martinsson, T., Weith, A., and Meuth, M. (1991): Genomic*

Nuclear oncoproteins affecting hematopoietic cell differentiation

Martin ZENKE	Group leader
Kim-Chew LIM	Visiting scientist
Petr BARTUNEK	Postdoc
Guido BOEHMELT	PhD-student
Karoline BRIEGEL	PhD-student
Christine DISELA	PhD-student
Gabi STENGL	Technician

Introduction

Our group has an ongoing interest to study oncogene function in leukemogenesis using retrovirus-transformed, primary chicken bone marrow cells as an experimental system. In addition, we investigate if and how nuclear proto-oncoproteins, nuclear hormone receptors and other transcription factors involved in gene regulation might affect hematopoietic cell differentiation and/or contribute to leukemogenesis if inappropriately or overexpressed in such cells.

Our research has over the last 2 years mainly focused on elucidating the molecular mechanism of action in erythroleukemia of the v-erbA oncogene which encodes a mutated thyroid hormone (T3/T4) receptor (type *a*). Therefore we have studied:

– the genetic alterations which turn the normal thyroid hormone receptor c-erbA into an oncoprotein (Zenke et al., 1990);

– v-erbA phosphorylation and its effects on v-erbA oncoprotein activity (Glineur et al., 1990);

– the erbA target gene Carbonic Anhydrase II (CAII; Disela et al., 1991b);

– whether there is a normal function of T3 and of the c-erbA/T3 receptor in red cell differentiation (Schroeder et al.,1991; see Report of Hartmut Beug).

It has been hypothesized that v-erbA acts as a dominant negative oncoprotein which antagonizes normal endogenous c-erbA/T3 receptor function in red cell differentiation (Zenke et al., 1990; Damm et al.,1989; Sap et al., 1989). To directly test this hypothesis, we have now coexpressed v-erbA and c-erbA in stably transformed erythroid cells to investigate if and under which conditions the v-erbA oncoprotein acts as a dominant repressor of c-erbA and whether this is important for its activity as an oncoprotein (Disela et al., 1991b).

To study a potential function of other oncoproteins in leukemia formation, various conditional versions of v-myc, v-fos and v-rel have been constructed by fusion with the estrogen receptor hormone binding domain, thereby generating v-oncER fusion proteins which place the oncoprotein activity under control of estradiol.[1] A similar approach has been chosen to investigate the function of the GATA transcription factors in hematopoietic cell differentiation.

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

Christine Disela, Petr Bartunek and Martin Zenke, in collaboration with Henk Stunnenberg, Heidelberg, Hartmut Beug, Vienna, and Björn Vennström, Stockholm

v-erbA is one of the two oncogenes contained in the Avian Erythroblastosis Virus (AEV) and encodes a mutated thyroid hormone (T3/T4) receptor (type *a*).

In AEV-induced erythroleukemia, v-erbA contributes to leukemic cell transformation by efficiently blocking differentiation of red cell progenitors and by suppressing transcription of erythrocyte-specific genes (Zenke et al., 1990, and references therein). We have shown before that a retrovirus-transduced c-erbA/T3 receptor when overexpressed in erythroid cells, effectively regulates erythroid cell differentiation in a hormone-responsive fashion: differentiation is blocked in the absence and induced in the presence of T3 (Zenke et al., 1990). Likewise, in these cells transcription of a set of erythrocyte-specific genes which are constitutively suppressed by v-erbA, is modulated by T3: suppressed in the absence and activated in the presence of T3.

This finding has led to the notion that v-erbA contributes to leukemogenesis by acting as a dominant repressor of c-erbA/T3 receptor function in erythroid cell differentiation (Damm et al., 1989; Sap et al.,1989; Zenke et al., 1990). To directly test the dominant repressor hypothesis in

leukemic cells and on the erbA target gene Carbonic Anhydrase II (CAII), we stably introduced c-erbA into v-erbA-expressing erythroblasts and studied the effects of the coexpressed v-erbA and c-erbA proteins on:

(I) erythroid differentiation,

(II) erythrocyte-specific gene expression, and

(III) on the activity of transiently transfected reporter gene constructs containing the CAII-specific erbA binding site (Disela et al., 1991b).

These experiments demonstrated that in stably transformed erythroblasts coexpressing the v-erbA oncoprotein and the c-erbA/T3 receptor at an approximately equimolar ratio, c-erbA activity is dominant over v-erbA: T3 efficiently induced erythroid differentiation in these cells thus overcoming the v-erbA-mediated differentiation arrest. Likewise, T3 activated CAII transcription as well as transient expression of a T3-responsive reporter gene containing the CAII-specific erbA binding site. However, a large excess of v-erbA was able to extinguish the c-erbA-dependent activation of the CAII reporter gene.

These data suggest that in leukemic cells overexpression of v-erbA is required to efficiently suppress endogenous c-erbA/T3 receptor function and that this appears to be an essential feature for v-erbA activity in erythroid cell transformation (see Disela et al., 1991b).

2. Searching for erbA-regulated genes.

Karoline Briegel and Martin Zenke

So far 3 erythrocyte-specific genes have been identified whose expression is subject to regulation by erbA (suppression by v-erbA and hormone-dependent regulation by the c-erbA/T3 receptor; Zenke et al., 1990). These erbA-regulated genes encode the erythrocyte-specific anion transporter band3, the erythroid-specific Carbonic Anhydrase II (CAII) and the enzyme Aminolevulinate Synthase (ALA-S, which catalyses the first and rate-limiting step in heme biosynthesis). Two of these genes (band3, CAII) have been reintroduced into v-erbA-expressing leukemic erythroblasts, showing that repression of these two genes is responsible for the v-erbA-induced changes in the medium requirements of these cells (Fuerstenberg et al., 1990; see also research report Hartmut Beug).

In addition, these studies demonstrated that regulation of CAII and band3 expression cannot account for all of the phenotypic changes induced by erbA. We therefore set up experiments aiming at identifying additional erbA-regulated genes which by themselves might serve a major regulatory function (eg. as transcription factors) in red cell differentiation. Preliminary experiments suggested to us that expression of the GATA transcription factors GATA-1, GATA-2, GATA-3 (see below) was not affected by erbA.

This prompted us to search for erbA-regulated genes using a differential cDNA cloning approach. Therefore transformed erythroblasts expressing various conditional T3-inducible erbA proteins were treated with T3, or not treated, and representative cDNA libraries were constructed. These were differentially screened with the respective cDNA probes and potentially erbA-regulated cDNA clones were isolated. These cDNA clones are currently being analyzed.

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

Guido Boehmelt and Martin Zenke, in collaboration with Hartmut Beug, Vienna, and Paula Enrietto, New York

v-rel belongs to a family of related DNA-binding proteins which encompass e.g. the transcription factor NFkB and the Drosophila embryonic polarity gene dorsal. Proteins of this gene family are involved in such diverse functions as cell proliferation and differentiation, immediate early responses in inflammation, or development (Gilmore, 1990).

A functional correlation between cytoplasmic/nuclear translocation and transcriptional activity has best been demonstrated for the subunits of NFkB (p50 and p65). The human c-rel protein (p85) is believed to follow a similar mechanism of activation, whereas in transformed cells the v-rel oncoprotein seems to be always located both in the cytoplasm and the nucleus (Morrison et al., 1991 and references therein). In addition, *in vitro* experiments demonstrated that the $p59^{v\text{-}rel}$ can form a heterodimeric complex with the p50 subunit of NFkB. This finding and additional observations (Ballard et al., 1990; Kieran et al., 1990), led to the idea that the v-rel oncoprotein might act as a dominant negative version of one (or more) of the NFkB subunits (like p65, p75 or p85/c-rel) and thereby exerts its transforming activity.

To approach v-rel oncogene function in transformation of chicken bone marrow cells, a conditional v-relER has been constructed by fusion of v-rel to the estrogen receptor hormone binding domain (ER).

We observed that in the presence of estradiol, v-relER readily transforms bone marrow cells in culture. Such v-relER transformed cells grew only in the presence of estradiol and if kept in the presence of hormone exhibited the phenotype and cell surface marker make-up of v-rel transformed cells. Withdrawal of hormone caused the cells to stop proliferating and also induced changes in morphology and expression of cell surface markers. Further experiments shall address the question whether in the absence of hormone and therefore in the absence of an active v-relER oncoprotein these cells will differentiate and what the nature of such, possibly more mature cells is.

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

Karoline Briegel and Martin Zenke, in collaboration with Day Engel, Chicago)

GATA factors have been defined as a class of sequence-specific DNA-binding proteins that recognize the common consensus sequence WGATAR and function as transcriptional activators both in vitro and in vivo (Orkin, S.H., 1990). In chicken, GATA factors are encoded by a multigene family (GATA-1, -2, -3) with each family member exhibiting a unique pattern of tissue-restricted expression (Yamamoto et al., 1990).

GATA-1 was originally identified as the first erythrocyte-specific transcription factor (Tsai et al., 1989) which is also expressed in two related hematopoietic lineages (megakaryocytes and mast cells).

GATA-2 is transiently expressed at high levels in immature erythroid cells, however its expression decreases in the course of cell maturation as soon as GATA-1 and -3 become upregulated. GATA-2 expression is also found in embryonic brain, liver and muscle whereas GATA-3 is highly expressed in T-lymphocytes and in the brain. It is therefore tempting to speculate that GATA-factors might play an important role in erythropoiesis. This has been clearly demonstrated for GATA-1 (Pevny et al., 1991).

In order to study GATA-2 and -3 function in erythroid cell differentiation, conditional GATA versions have been constructed by fusion with the estrogen receptor hormone binding domain (in the following refered to as GATA/ER). Such hybrid GATA/ER proteins efficiently enhanced transcription of a reporter gene construct in transient transfection experiments in a hormone-responsive fashion (K. B. and K.-C. L., unpublished). As a next step, the GATA/ERs will be introduced into primary chicken bone marrow cells via recombinant retrovirus vectors. We will then study the effects of GATA/ER expression in the presence and absence of estradiol on red cell differentiation.

PUBLICATIONS SUBMITTED 1991

1. *Disela, C., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., and Zenke, M. (1991b): v-erbA overexpressison is required to extinguish c-erbA function in erythroid cell differentiation and regulation of the erbA target gene CAII. Genes & Development* **5**, *2033–2047.*
2. *Schroeder, Ch., Gibson, L., Zenke, M., and Beug, H. (1991): Modulation of normal erythroid differentiation by the endogenous thyroid hormone- and retinoic acid receptors: a possible target for v-erbA oncogene action. Oncogene, in press.*
3. *Beug, H., Doederlein, G., and Zenke, M. (1991): Transformation by v-erbA and v-erbB oncogenes: Independent modulation of differentiation and proliferation in erythroid progenitors. In Bristol Myers Symposia on Nuclear Processes and Oncogenes, Vol. 15 (P. A. Sharp, ed.) Academic Press Inc., Orlando, Florida, in press.*
4. *Zenke, M. (1991): v-erbA., in Guidebook to Oncogenes; Marshall, C., Müller, R., and Ozanne, B. (eds.), Sambrook and Tooze Scientific Publishers, in press.*
5. *Quarto, R., Dozin, B., Tacchetti, C., Robio, G., Zenke, M., Campanile, G., and Cancedda, R. (1992): Constitutive myc expression impairs hypertrophy and calcification in cartilage. Dev. Biol.* **149**, *168–176.*
6. *Morrison, L., Boehmelt, G., Beug, H., and Enrietto, P. (1991): Expression of v-rel in a replication competent virus: transformation and biochemical characterization. Oncogene* **6**, *1657–1666.*
7. *Disela, C., Walter, C., Dodgson, J., Beug, H., and Zenke, M. (1991a): The v-erbA oncoprotein efficiently suppresses erythroid-specific CAII expression. In: Carbonic Anhydrase: From Biochemistry and Genetics to Physiology and Clinical Medicine. Edited by Botre, F., Gros, G., and Storey, P. T., VCH, Weinheim, p. 139–143.*

OTHER REFERENCES

Ballard et al. (1990): Cell **63**, *803–814.*
Damm et al. (1989): Nature **339**, *593–597*
Fuerstenberg et al. (1990): J. Virol. **64**, *5891–5902.*
Gilmor (1990): Cell **62**, *841–843 (1990).*
Glineur et al. (1990): Genes Dev. **4**, *1663–1676.*
Kieran et al. (1990): Cell **62**, *1007–1018.*
Orkin (1990): Cell **63**, *665–672.*
Pevny et al. (1991): Nature **349**, *257–260.*
Sap et al. (1989): Nature **340**, *242–244.*
Tsai et al. (1989): Nature **339**, *446–451.*
Yamamoto et al. (1990): Genes Dev. **4**, *1650–1662.*
Zenke et al. (1990): Cell **61**, *1035–1049.*

Animal House

Erwin WAGNER	Scientific coordinator
Karl SCHELLANDER	Veterinary doctor (University Vienna)
Norma HOWELLS	Chief technician
Gerhard JURITSCH	Technician
Mijo DEZIC	Technician
Erika PENNINGER	Technician

The animal house provides technical support to the scientists and at present holds various in and out-bred, as well as hybrid strains of mice, random-bred rabbits, out-bred chickens and random-bred *Xenopus laevis* toads. During the last year, the demand for mice for experimental studies has risen by 15%, subsequently, we have intensified the breeding programs and also introduced several more strains. Current production of animals bred in-house is as follows: approx. 275 mice/week, 10 chicken embryos/week and 12 chickens/week. We have continued to rear our own *Xenopus laevis* toads (to date 170). These have been a useful addition (despite their slow growth rate, i.e. 2–5 years to maturation) to those purchased from external sources. Rabbits are still purchased from external suppliers for economic reasons. 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 Harmut Beug and Martin Zenke); and *Xenopus laevis* studies (see Report of Max Birnstiel).

Technical procedures offered by the animal house are performed to a high standard, thereby ensuring that any discomfort to the animal is kept to a minimum. These include collection of rabbit and chicken blood, administration of hormones to mice, tumour implantations on mice and chicks, hysterectomy and subsequent resuscitation of potential transgenic and chimaeric mice, collection of tissue such as skin, muscle, liver, spleen, etc. and embryonic tissue for DNA and RNA analysis by the scientists.

The introduction of a negative pressure isolator has enabled us to facilitate the use of mice for possible infective murine virus studies and to receive mice which may not be up to the required health standard for our animal-house. In addition, we have recently acquired two Scantainers-(mobile ventilated cabinets), particularly for studies requiring more protection, for example an immune-depressed nude mice breeding colony.

Computer Group (Protein Modelling)

Anton BEYER	Scientist
Aron BAUER	PhD-student
Georg CASARI	PhD-student (January to April 1991)
Eva SILHAVY	Diploma student (January to May 1991)

Introduction

Our main interest is the relationship between amino acid sequence and tertiary structure of globular proteins. There are three main topics we are currently working on.

Using purely empirical data from known crystal structures one can obtain a potential of mean force. This method was developed by Manfred Sippl in Salzburg and extended and thourougly investigated by Georg Casari during his thesis which he worked on during his stay at the IMP from 1988 to 1991. Currently this work is continued by Aron Bauer in collaboration with Manfred Sippl and Georg Casari in Salzburg.

In a different approach we are using empirical energy functions for energy minimization, molecular dynamics calculations and simulated annealing for conformational calculations on phospholipase A2 (Aron Bauer) and Lysozyme T4 (Eva Silhavy).

Because the availability of all sorts of structural data is a prerequisite for protein modelling and structure prediction we are using a relational database system for storing these data in a compact and consistent form. This information can then easily be used for protein modelling projects.

Development of Optimization Methods for Protein Structure Prediction

Aron Bauer

Currently about 600 protein structures are known in detail from x-ray analysis of protein crystals or NMR-studies of proteins in solution. One way of utilization of all this information is constructing a potential of mean force (PMF) from statistical analysis of interatomic distances via an inverse Boltzmann law. Manfred J. Sippl outlined the method (1) and has furthermore shown that the PMF allows a clear discrimination between native protein structures and misfolded ones (2).

The quality of the PMF depends strongly on the selection of an adequate set of reference proteins. We found that it is very sensitive to errors in the coordinates and to the use of homologous structures. Furthermore proteins with very large prosthetic groups tend to distort the PMF. Therefore a limited set of about 100 structures was used for calculations.

Since the PMF can be used as a criterion to identify native folds, we are looking for methods to generate and optimize structures from scratch, starting with the protein sequence alone. Currently two strategies are under investigation: Monte Carlo methods (MC) and Genetic Algorithms (GA).

The monte carlo algorithm we use differs from the classical Metropolis scheme. It is a force-biased variant of the so called "Sintflut-Algorithmus" proposed by G. Dück (ISAM, Heidelberg), which is an analogy to simulated annealing. We have modified the method to allow crossing of small energy barriers and ensure a more homogeneous distribution of energy throughout the structure. This method works fine on n-dimensional hypersurfaces when there are n independent contributions to the potential function, but still faces problems with the highly interdependent PMF for protein chains.

The genetic algorithms we use so far are very simple variants of the scheme proposed by Holland (3). The main problem we encounter is that the efficiency of selection is too high and leads to a sharp distribution of fitness in the population, therefore decreasing the size of the "genepool". This quickly creates a situation where an increase in fitness can be achieved by mutations only, which is equal to a pure random search.

Our main goals are the improvement of these methods and the construction of a more general constraint satisfaction algorithm (4), which will use the PMF for constraint generation and utilize further information than the primary structure (e.g. a library of possible supersecondary structures).

REFERENCES

1. *Sippl, M. J. (1990): J. Mol. Biol.* ***213****, 859–883.*
2. *Hendlich, M., Lackner, P., Weitckus, S., Flöckner, H., Froschauer, R., Gottsbacher, K., Casari, G., and Sippl, M. J. (1990): J. Mol. Biol.* ***216****, 167–180.*
3. *Sumida, B. H., Houston, A. I., Mc Namara, J. M., and Hamilton, W. D. (1990): J. Theor. Biol.* ***147****, 59–84.*
4. *Major, F., Turcotte, M., Gautheret, D., Lapalme, G., Fillion, E., and Cedergren, R. (1991): Science* ***253****, 1255–1260.*

Conformational Calculations on Phospholipase A2

The known three-dimensional structures of bovine and porcine Phospholipase A2 (PLA2) (1) provide a striking example of how local protein structure may change due to single amino acid mutations (2). The enzymes are highly homologous and almost identical in tertiary structure, but a surface loop of 13 amino acids adopts different conformations due to a substitution of VAL63 by PHE in the middle of the loop.

Using a classical molecular forcefield (GROMOS) we perform molecular dynamics simulations for a mutant protein with the structure of one and the sequence of the other PLA2 molecule. To reduce the size of the system, a spherical shell was cut from the molecule that contains the region of interest and a boundary zone larger in diameter than the non-bonded cutoff. The atoms in the boundary zone are held in place by applying a restraining potential.

The molecular dynamics is performed at variable temperatures, where large mobility is observed at high temperatures and a quick decrease in potential energy occurs during cooling in a narrow temperature range.

The big variety of obtained conformations focused our attention on the size of the conformational space that is accessible to this surface loop. Indeed this part of the protein has been described to be rather flexible (1). This flexibility might also play a central role in binding the enzyme to the surface of micelles, where catalytic activity naturally occurs.

To explore the size of conformational space we calculate molecular dynamics trajectories of the system described above at high temperatures (700–800 K). Every 10 picoseconds the intermediate structure is minimized using a conjugate gradient algorithm.

The obtained conformations are compared to the native structures and classified according to their backbone dihedral angles by a cluster analysis method. After less than 2 ns simulation time all newly generated conformations fall into preliminary defined classes of structures.

REFERENCES

1. *Dijkstra, B. W., Renetseder, R., Kalk, K. H., Hol, W. G. J., and Drenth, J. (1983): Structure of Porcine Pancreatic Phospholipase A2 at 2.6 Angstroem Resolution and Comparison with Bovine Phospholipase A2. In J-Mol-Biol,* ***168****, 163–179.*
2. *Kabsch, W., and Sander, C. (1984): On the use of sequence homologies to predict protein structure: Identical pentapeptides can have completely different conformations. In: Proc. Natl. Acad. Sci. USA,* ***81****, 1075–1078.*

Structural Database for Proteins

Anton Beyer

The availibility of and easy access to structural information of various kinds, like cartesian coordinates, secondary structure assignment based on x ray data or NMR experiments is very important for protein modelling projects. Currently the main source of these data is the Brookhaven Protein Databank (1). The organization of the data in more or less independent datasets for each protein is not very convenient for questions concerning more than one single protein. In the last years several attempts have been made to use the concept of a relational database for addressing this sort of questions (2,3). We decided to use a commercially available database system (ORACLE) to build a structural data base for proteins. The benefits of such a system are consistency of the data, which indeed is very important for statistical investigations, and an English like query language for questions concerning different aspects of the stored information. In a relational database all information is kept in tables. We are using tables for general information of each dataset, sequence information, secondary structure tables and one table for cartesian coordinates. Currently we are using all datasets from the Protein Databank with a resolution of less or equal two Angstroems. We will use all available datasets in the near future. Storing the information in a consistent way and easy access by directly formulating questions is one of the most important benefits of such a database.

REFERENCES

1. *Berstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, D. F., Jr., Brice, M.D., Rodgers, J. R., Kennard, O., Shimanuchi, T., and Tasumi, M. (1977): Protein data bank: A computer-based archival file for macro-molecular structures. J. Mol. Biol.* ***112****, 5535–5542.*
2. *Islam, S. A., and Sternberg, M. J. E. (1989): A relational database of protein structures designed for flexible enquiries about conformation. Protein Eng.* ***2****, 431–442.*
3. *Huysmans, M., Richelle, J., and Wodak, S. J. (1991): SESAM: A Relational Database for Structure and Sequence of Macromolecules. Proteins* ***11****, 59–76.*

Outlook

Different methodes are used for protein structure predictions which is the main topic of our work. The following project are ongoing efforts along these lines:

A. Beyer
Design and implementation of a structural database for proteins using the relational database concept.

A. Bauer
Development of Optimization methods for Protein Structure Prediction.

A. Bauer
Conformational Calculations on Phospholipase A2

E. Silhavy
Conformational calculations and structural predictions of point mutations of lysozyme

PUBLICATIONS SUBMITTED 1991

1., *Beyer, A., Kalchhauser, H., and Wolschann, P. (1991): Molecular Modeling on Garuganin-I. Monatshefte für Chemie. Submitted.*

Service Department

Gotthold SCHAFFNER	Scientist
Sissy AIGNER	Technician (until June 1991)
Ivan BOTTO	Technician
Jiři DOŠKAŘ	Visiting scientist
Robert KURZBAUER	Technician
Andrea SCHNATTINGER	Postdoc (since July 1991)

The Service Department is going into its fifth year and the idea of providing a variety of instant services is accepted more than ever among the IMP scientists. Our most popular services are DNA sequencing and oligonucleotide synthesis. In addition to these time consuming services, we also test titers of antisera and discuss immunization protocols with the scientists and the Animal House staff, in order to find the optimal way of raising antibodies against a particular protein or oligopeptide.

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

Oligonucleotide Synthesis

This year we again synthesized greater than 1200 oligonucleotides on the APPLIED BIOSYSTEMS 380 B. The demand for PCR primers increased, whereas less short primers were synthesized because of the change in our sequencing strategy (EXO III deletion clones instead of "primer walking"). A small proportion of oligo-nucleotides were synthesized with modified 3' or 5' – ends (e.g. an amino group for further coupling to a solid support or to biotin).

Direct coupling of a biotin-phosphoramidite and phosphorothioate oligonucleotidesynthesis were performed on the PHARMACIA GENE-ASSEMBLER because of lower valve-blocking risk. These syntheses were performed by, or in close collaboration with, Berndt Oberhauser.

Synthesis of methoxy- or ethoxy-RNA oligonucleotides has now become as easy as DNA oligonucleotide synthesis. The coupling efficiency being dependent upon the quality of the monomers only and can be as high as in DNA synthesis.

Sequencing

The bulk of the 3000+ DNA probes sequenced were crude double stranded DNA's isolated by either alkaline lysis or the boiling preparation method. We have made some studies with regard to primer design and have found that in conventional sequencing, using 32 P – dATP and the SEQUENASE (USB) protocol with T7 DNA POLYMERASE (PHARMACIA) the amount of Adenosine in the sequence immediately after the 3' end of the primer has a major influence on the signal and seems to be as important as the amount of DNA used. On the other hand, we found no simple rule for primers used with the dye dideoxy terminator system and TAQ polymerase for automated sequencing with the APPLIED BIOSYSTEMS 373 A; the results being as good as those obtained by conventional sequencing. In parallel experiments we have used the ABI 373 A results to correct those read from the autoradiograms and vice versa. ABI 373 A data of GC-rich sequences are easy to read in contrast to the corresponding "strong stops" on the autoradiograms. At the moment we are using the 373 A mainly for sequencing DNA purified from CsCl gradients, however, we have also successfully used the ABI 373 A with crude DNA as well. DNA synthesized by PCR and then isolated from agarose gels has also been sequenced successfully ("direct sequencing"). To improve this procedure we have started to use biotin labelled primers which allows us to isolate the DNA with streptavidine loaded magnetic beads (DYNAL).

During late spring we started a sequencing project with Jiri Doskar, from BRNO, Tchechoslovakia, who was awarded a UNESCO/TWAS felloship for a 3 month-training period in our laboratory.

A 40 kbp phage of Staphylococcus aureus was subcloned, deletion clones of a 4.6 kbp subclone were made and sequenced.

As the capacity of the ABI 373 A is far too low, we have only sequenced about 20 kbp with it, the bulk of sequencing still beeing done the conventional way. We are currently evaluating systems which can read autoradiograms accurately so as to reduce the time required to produce the final sequence.

PUBLICATIONS SUBMITTED 1991

1. *Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, Ch. R., Schaffner, G., Wagner, E., and Birnstiel, M. L. (1991): 2'-0-Methyl, 2'-0-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribonucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event. Nucl. Acids Res.* ***19****, 2629–2635.*
2. *Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M., and Noe, Ch. R. (1991): A simple procedure for the preparation of protected 2'0-methyl or 2'0-ethyl ribonucleoside-3'-0-phosphoramidites. Nucl. Acids Res.* ***19****, 5965–5971.*
3. *Smith, O., Tabiti, K., Schaffner, G., Soldati, D., Albrecht, U., and Birnstiel, M. L. (1991): Two-step affinity purification of U7 small nuclear ribonucleoprotein particles using complementary biotinylated 2'0-methyl oligoribonucleotides. Proc. Natl. Acad. Sci. USA* ***88****, 9784–9788.*
4. *Phillips, St., Cotten, M., Laengle-Rouault, F., Schaffner, G., and Birnstiel, M. L. (1991): Amphibian oocytes and sphere organelles: are the U snRNA genes amplified? Submitted to Chromosoma.*

The PR-office

Martina STEINHARDT, PR-speaker

The PR-office has to fulfil a variety of tasks required by the public. This year, 1991, was ruled by the slogan: "Austria needs a law on genetic engineering." This encouraged many people, private and professional, to ask for appropriate information. It is essential that the law rules be communicated to the public and made sure the context is understood. A questionnaire worked out by the PR-office Hargitay & List indicated that in general the public agrees to gene technological work within defined limits, and that most fears come from too little or false information. In this field there is still much left to be done in 1992 for scientists as well as PR-speakers.

Interviews and discussions with journalists

This year the discussion forum developed into a seriously working group of people interested in informing the public. After three general meetings on different topics the group focused on the discussion about the preliminary concept of a "gene-technology-law" in March 1991. This version, established by the Ministry of Health showed a definite lack of scientific background and asked for more biologists to participate in formulating the new law.

Apart from this, important work has been done with the journalist Helmuth Santler in the field of health education. Every year the PHARMIG community donates a prize of AS 50,000.– for the best documentations on health education. Mr. Santler was given advice and offered co-operation with the PR office on the topic, how gene diagnostics could benefit to individual health care. Health insurances should be discouraged from discriminating people with genetic disorders by higher fees but help them increase their life quality by regular physical examinations.

Visiting groups

In 1991, 28 groups came to visit I.M.P. 9 of them were high school students and teachers with one student writing a scientific essay on basic cancer research for her university entry examination. This year many medical specialists either came for general information, for advice or for co-operation with I.M.P. The PR-office organized guiding tours, contacts with I.M.P. scientists and provided the information required about our work.

Representation of I.M.P. in the public

As mentioned in the introduction, much work is still to be done to communicate the possibilities and risks of genetic engineering to the public. According to the questionnaire, most people (up to 92 %) feel too little informed, which should be a challenge for any scientific PR office to fill this gap. Not only was the I.M.P. circular "**I**nfo**M**edien**P**resse" established but also several public seminars attended, mainly about the new law on genetic engineering:

– the genetech hearing at the Wilhelminenberg;

– the podium discussion with Jeremy Rifkin (Renner-Institute, Vienna) about potential risks of gene technology;

– regular PHARMIG – PR-meetings, organized by the chairwoman of PHARMIG, Mrs. Mayrhofer;

– the SANDOZ journalists' seminar, organized by PHARMIG about cancer and AIDS.

PUBLICATIONS

Steinhardt, M.: Gentechnologie in der Krebsforschung, CliniCum Mai 1991, pp. 24–25.

Steinhardt, M.: Erbkrankheiten – eine heikle Frage, CliniCum Juli/August 1991, pp. 24–26.

Steinhardt, M.: Menschen 2. Klasse durch Gendiagnostik? CliniCum Oktober 1991, pp. 22–24.

*Steinhardt, M.: Schwerpunkte in der Krebsforschung, ÖAZ **45**/31–32 (1991), pp. 650–651.*

*Steinhardt, M.: Gendiagnostik – 2 Seiten eines Talers, ÖAZ **45**/44 (1991), p. 960.*

*Steinhardt, M.: Krebs-Lichtblicke, AUSTRIA INNOVATIV **2**/1991, p. 33.*

Diploma- and PhD-Theses of I.M.P. Students in 1991

Aron Bauer (Group A. Beyer)
Diploma thesis: "Punktmutation in Proteinen. Konformationssuche mittels simulated annealing und Molekulardynamik am Beispiel der Phospholipase A2", submitted to the University of Vienna.

Georg Casari (Group A. Beyer)
PhD-thesis: "Potentiale der mittleren Kräfte in Proteinen. Analyse und Ableitung physikalischer Prinzipien", submitted to the University of Vienna.

Christine Disela (Group M. Zenke)
PhD-thesis: "Regulation der Erythrozyten-spezifischen Carboanhydrase durch das v-erbA Onkoprotein", submitted to the University of Marburg.

Richard Eckner (Group M. L. Birnstiel)
PhD-thesis: "Nucleo-cytoplasmic transport of mRNA: the formation of histone RNA 3' ends and the export of histone mRNA are interdependent events", submitted to the University of Zurich.

Irene Leitner (Group H. Beug)
Diploma thesis: "Reexpression von Carboanhydrase II in v-erbA-enthaltenden Hühner-Erythroblasten", submitted to the University of Vienna.

Claudia Petritsch (Group G. Ammerer)
Diploma thesis: "Modification of the transcription factor STE12 in the pheromone response pathway", submitted to the University of Vienna.

Wilfried Rossol (Group T. Schuster)
Diploma thesis: "MST1 and MST2: Two homologous genes encoding putative membrane spanning proteins".

Shantini Sockanathan (Group K. Nasmyth)
PhD-thesis: "Characterization of SWI4: a yeast cell-cycle specific transcriptional activator", submitted to the University of Cambridge, UK.

Norbert Schweifer (Group T. Schuster)
Diploma thesis: "Molecular and genetic analysis of the plasminogen locus in wild type and t haplotype forms of mouse chromosome 17", submitted to the University of Vienna.

Alain Vasserot (Group M. L. Birnstiel)
PhD-thesis: "Biochemistry of histone RNA 3' end formation", submitted to the University of Zurich.

I.M.P SEMINAR LIST 1991

04.01.91:	**JAMES MANFREDI** (Columbia Univ.): "Role of p53 and pRB in SV40 large T antigen-mediated oncogenesis"
09.01.91:	**JEFF SCHATZ**, (Biozentrum Basel): "The protein import machinery of mitochondria"
24.01.91	**CHRIS HIGGINS** (ICRF, Oxford): "Molecular mechanisms of membrane transport: from microorganisms to multidrug resistance and cystic fibrosis"
08.02.91:impromptu	**AXEL RETHWILM** (Würzburg): "Molecular biology of the human foamy virus"
12.02.91: impromptu	**MANFRED NEUBERG** (Marburg): "Mechanisms of *fos* induced transformation"
14.02.91	**DAVID PORTEOUS** (Edinburgh): "Human genome analysis by targetted recombination and coincident sequence cloning"
21.02.91	**KURT WÜTHRICH** (Zürich): "Protein-DNA interactions studied by NMR in solution: Homeodomains and repressors"
22.02.91: impromptu	**THOMAS JENUWEIN** (UCSF): "Activation and expression of immunoglobulin m chain in transgenic mice"
27.02.91: impromptu	**MICHAEL FREISMUTH** (Vienna): "G proteins: structural determinants for GTP hydrolysis and receptor interactions"
28.02.91	**ROBB KRUMLAUF** (NIMR, London): "Homeobox genes and pattern formation in vertebrates"
07.03.91	**KURT BALLMER-HOFER** (FMI, Basel): "Regulation of cellular tyrosine kinases by polyomavirus middle-T antigen"
14.03.91	**UELI SCHIBLER** (Geneva): "Genes controlling transcription and proliferation during liver differentiation"
21.03.91: impromptu	**MICHAEL WILES** (Basel I.for Immunol.): "Haematopoietic lineages from ES cells *in vitro* and *in vivo*"
04.04.91	**FRANCOIS CUZIN** (Nice, France): "Expression of polyoma large T in the testes of transgenic mice and the subsequent establishment of "differentiated" cell lines"
09.04.91: impromptu	**FRANZ-ULRICH HARTL** (Munich) "The role of the chaperonin hsp60 in protein folding in mitochondria"
10.04.91:	**ANDRAS NAGY** (Toronto): "Pluripotentiality of embryonic stem cells"
18.04.91	**GÜNTHER SCHÜTZ** (DKFZ, Heidelberg): "Extinction of gene activity by interference with cAMP signal transduction"
23.04.91: impromptu	**KARL-HEINZ KLEMPNAUER** (Freiburg-Zähringen): "Regulation of gene expression by *myb*- and *myb*-related genes"
25.04.91	**KLAUS RAJEWSKI** (Cologne) "Gene targeting in the analysis of B cell development"
26.04.91: impromptu	**DOUG ENGEL**.(Illinois): "GATA transcription factor gene regulation in development"
16.05.91	**CARL-HENRIK HELDIN** (Uppsala, Sweden) "Structural and functional properties of growth regulatory molecules from human platelets"
05.06.91	**ELI GILBOA** (New York): "Intracellular immunization against HIV using RNA decoys"
06.06.91	**RICHARD TREISMAN** (ICRF, London): "Structure and function of SRF"
07.06.91: impromptu	**DOUGLAS HANAHAN** (UCSF): "Characterizing the stages of multistep tumorigenesis pathways in transgenic mice"

13.06.91	**CHRIS MARSHALL** (Chester Beatty, London): "p21 *ras* signals for transformation and membrane localization"
27.06.91	**MICHAEL NEUBERGER** (MRC, Cambridge): "Immunoglobulin gene expression"
03.07.91	**PROF. ANTON PREISINGER** (TU, WIEN): "Strukturchemische Aspekte der Hydrolyse von ras p21.GTP.Mg"
04.07.91	**REGINE KAHMANN** (IGF Berlin GmbH): "A molecular analysis of pathogenicity in *Ustilago maydis*"
11.07.91	**JOHN SKEHEL** (NIMR, London): "Membrane fusion by influenza haemagglutinin"
05.08.91: impromptu	**DAVE BRAMHILL** (Rahway, New Jersey): "The mechanism of initiation of DNA replication at the *E. coli* chromosomal origin"
08.08.91	**WIEBE KRUIJER** (Utrecht): "jun/Ap1 and EC differentiation"
14.08.91	**MARK DWORKIN** (Vienna): "Regulation of carbon metabolism during embryogenesis and tumorigenesis"
28.08.91: impromptu	**MICHELLE MILLER** (Sydney): "v-*erb*-B-mediated transformation as a model for leukaemia development"
30.08.91: impromptu	**MICHAEL KARIN** (UCSD): "Positive and negative regulation of AP-1 activity - the Ying-Yang of cell proliferation"
02.09.91: impromptu	**TADATSUGU TANIGUCHI** (Osaka, Japan): "Regulation of the type I interferon system by DNA binding factors, IRF-1 and IRF-2"
03.09.91: impromptu	**BRANKO STEFANOVIC** (Tallahassee, Fl): "Developmental regulation of the sea urchin U2 snRNA genes"
05.09.91	**RICHARD GARDNER** (ICRF, Oxford): "Cell lineage and the stability of differentiation in mammalian development"
13.09.91: impromptu	**JOSEPH SCHLESSINGER** (NYU Medical Center): "Signal transduction by EGF-Receptor tyrosine kinase"
19.09.91	**NICK HASTIE** (Edinburgh): "Wilms tumour and aniridia – human developmental mutations and mouse model systems"
20.09.91	**UNA CHEN** (Basel Inst. f. Immun.): "Some evidence of lymphocyte development from mouse ES cells *in vitro*"
26.09.91 – 28.09.91:	**I.M.P WORKSHOP 1991**
03.10.91	**ROLF ZELLER** (EMBL): "The role of formins in vertebrate pattern formation"
10.10.91	**TOBY GIBSON** (EMBL, Heidelberg): "Structure modelling studies on DNA binding domains of transcription factors"
15.10.91: impromptu	**FRITZ PROPST** (Ludwig Inst., London): Phenotypes caused by the Mos oncogene in transgenic mice and transformed cells.
17.10.91	**GUNNAR VON HEIJNE** (Huddinge, Sweden): "How membrane proteins insert into membranes"
21.10.91: impromptu	**ROBERT MURPHY** (Pittsburgh): "Endosomal pH regulation and lysosome biogenesis"
22.10.91	**JOHN GURDON** (Cambridge): Muscle gene activation following embryonic induction in *Xenopus* embryos"
24.10.91	**ROSA BEDDINGTON** (Edinburgh): The notochord: A central influence during early pattern formation in the mouse embryo"
07.11.91	**PAOLO SASSONE-CORSI** (Strasbourg): "Transcription factors as final targets of signal transduction pathways"
14.11.91	**LEWIS WOLPERT** (London): "Pattern formation in development"

21.11.91	**BERNHARD DOBBERSTEIN**: (EMBL): "Signals and receptors involved in protein insertion into the membrane of the endoplasmic reticulum"
28.11.91	**ERNST HAFEN** (Zürich): "Specification of cell fate in the developing eye of *Drosophila*"
05.12.91	**WALTER SCHAFFNER** (Zürich): "Different factor domains stimulate transcription from a (remote) enhancer or a (proximal) promoter position"
12.12.91	**MARGARET BUCKINGHAM** (Paris): "Myogenesis in the mouse." Studies of Studies of
19.12.91	**CLAUDIO BORDIGNON** (Milan): "Gene therapy for human immunodeficiencies. Reconstitution of immune specific functions and T-cell repertoire."

I.M.P. PATENT APPLICATIONS 1991

CASE 12/117
"Olikonukleotide, die für ein modifiziertes aktives Zentrum der HRV2 2A kodieren, wobei die Modifikationen die "Cisaktivität" der Proteinase beeinflussen, vorzugsweise inhibieren, sowie die von abgeleiteten Expressionsplasmide"
Meldung der Diensterfindung: 15. Oktober 1990 (Bender)
4. Jänner 1991 (I.M.P.)
Annahme vom I.M.P.: 10. Jänner 1991
Erfinder: G. Casari (D. Blaas, E. Küchler, T. Skern, W. Sommergruber, M. Schreiber)

CASE 14/007
"Neue, über Endozytose in höhere eukaryotische Zellen aufnehmbare, Nukleinsäure enthaltende Komplexe"
Meldung der Diensterfindung: 19. März 1991
Annahme vom I.M.P.: 19. März 1991
Erfinder: M. L. Birnstiel, M. Cotten, E. Wagner

CASE 14/008
"2'-O-Alkyl-oligoribonukleotide, Verfahren zu deren Herstellung und deren Verwendung als Antisense-Oligonukleotide"
Meldung der Diensterfindung: 20. August 1991
Annahme vom I.M.P.: 21. August 1991
Erfinder: M. L. Birnstiel, M. Cotten, B. Oberhauser, G. Schaffner, E. Wagner
(H. Brunner, TU; A. Holzner, TU; G. Issakides, TU; M. Knollmüller, TU; Ch. Noe, TU;)

CASE 14/009
"Neue Protein-Polykation-Konjugate"
Meldung der Diensterfindung: 4. Juni 1991
Annahme vom I.M.P.: 5. Juni 1991
Erfinder: M. L. Birnstiel, M. Cotten, E. Wagner

CASE 14/010
"Neue Protein-Polykation-Konjugate"
Meldung der Diensterfindung: 4. Juni 1991
Annahme vom I.M.P.: 5. Juni 1991
Erfinder: M. L. Birnstiel, M. Cotten, E. Wagner

CASE 14/011
"Neue Konjugate, bestehend aus einem Glykoprotein und einer Nukleinsäure-bindenden Substanz"
Meldung der Diensterfindung: 7. August 1991
Annahme vom I.M.P. 8. August 1991
Erfinder: M. L. Birnstiel, M. Cotten, E. Wagner

CASE 14/012
"Steigerung der Transferrinfektion durch Beigabe von inaktivierten Viren"
Meldung der Diensterfindung: 7. November 1991
Annahme vom I.M.P. 25. November 1991
Erfinder: M. L. Birnstiel, M. Cotten, Ch. Plank, E. Wagner (D. Curiel, K. Zatloukal)

CASE 14/013
"Neue, Adenoviren enthaltende Konjugate für effizienten Gentransfer"
Meldung der Diensterfindung: 7. November 1991
Annahme vom I.M.P. 25. November 1991
Erfinder: M. L. Birnstiel, M. Cotten, Ch. Plank, E. Wagner (D. Curiel, P. C. Hu, K. Zatloukal)

CASE 14/014
"Neue, Adenovieren enthaltende Konjugate für den Transfer von Nukleinsäuren in eukaryotische Zellen"
Meldung der Diensterfindung: 7. November 1991
Annahme vom I.M.P. 25. November 1991
Erfinder: M. L. Birnstiel, M. Cotten, Ch. Plank, E. Wagner (D. Curiel, K. Zatloukal)

CASE 14/015
"New recombinant Baculoviruses"
Meldung der Diensterfindung: 29. November 1991
Annahme vom I.M.P. 2. Dezember 1991
Erfinder: K. Nasmyth, (Dr. N. Jones, Dr. G. Patel)

PUBLICATIONS 1991

1. Aguzzi, A., Kleihues, P., Heckl, K., and Wiestler, O. D. (1991): Cell-type specific tumor induction in neural transplants by retrovirus-mediated oncogene transfer. Oncogene **6**, 113–118.

2. Alexander, W., Lyman, S., and Wagner, E. F. (1991): Expression of functional c-kit receptors rescues the genetic defect of W mutant mast cells. EMBO J. **10**, 3683–3691.

3. Artzt, K., Barlow, D., Dove, W., Fischer-Lindahl, K., Lyon, M. F., Klein, J., and Silver, L. M. (1991): Mouse Chromosome 17. Mammalian Genome **1**, 5280–5300.

4. Ballou, L. M., Luther, H., and Thomas, G. (1991): MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways. Nature **349**, 348–350 (1991).

5. Barlow, D. P., Stöger, R., Saito, K., Herrmann, B. G., and Schweifer, N. (1991): The mouse Insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature **349**, 84–87.

6. Beug, H., and Vennström, B. (1991): Avian erythroleukemia: Possible mechanisms involved in v-erbA oncogene function. In: Nuclear Hormone Receptors (ed. Parker, M. D.), Academic Press Limited, London, pp. 355–375.

7. Bothe, K., Aguzzi, A., Lassmann, H., Rethwilm, A., and Horak, I. (1991): Progressive encephalopathy and myopathy in transgenic mice expressing human foamy virus genes. Science **253**, 555–557.

8. Boulter, C. A., Aguzzi, A., Williams, R. L., Wagner, E. F., Evans, M. J., and Beddington, R. (1991): Aberrant development and twinning induced by v-*src* in chimaeric mouse embryos. Development **111**, 357–366.

9. Burkert, U., von Rüden, T., and Wagner, E. F. (1991): Early fetal hematopoietic development from *in vitro* differentiated embryonic stem cells. The New Biologist **3**, 698–708.

10. Committee* for the mouse chromosome 17: Maps of mouse chromosome 17; First Report.
* Committee: Artzt, K., Barlow, D. P., Dove, W. F., Fischer-Lindahl, K., Klein, J. (chairman), Lyon, M. F., and Silver, L. M. (1991): Mammalian Genome **1**, 5–29.

11. Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, Ch. R., Schaffner, G., Wagner, E., and Birnstiel, M. L. (1991): 2'-0-Methyl, 2'-0-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribo-nucleotides as inhibitors of the *in vitro* U7 snRNP-dependent mRNA processing event. Nucl. Acids Res. **19**, 2629–2635.

12. Cox, R. D., Weydert, A., Barlow, D., and Buckingham, M.E. (1991): Three Linked Myosin Heavy Chain Genes Clustered Within 370 kb of Each Other Show Independent Transcriptional and Post-transcriptional Regulation during Differentiation of a Mouse Muscle Cell Line. Developmental Biology **143**, 36–43.

13. Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991): Adenovirus enhancement of transferrin-polylysine mediated gene delivery. Proc. Natl. Acad. Sci. USA **88**, 8850–8854.

14. Dirick, L., and Nasmyth, K. (1991): Positive feedback in the activitation of G1 cyclins in yeast. Nature **351**, 754–757.

15. Disela, Ch., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., and Zenke, M. (1991): v-*erb*A overexpression is required to extinguish c-*erb*A function in erythroid cell differentiation and regulation of the *erb*A target gene CAII. Genes & Development **5**, 2033–2047.

16. Disela, Ch., Walter, C., Dodgson, J., Beug, H., and Zenke, M. (1991): The v-*erb*A Oncoprotein efficiently suppresses erythrocyte-specific CA II Expression. Proceedings on carbonic anhydrases. Edited by Botrè, F., Gros, G., and Storey, B.T., pp. 139–143.

17. Eckert, W. A., Plass, C., Weith, A., Traut, W., and Winking, H. (1991): Transcripts from amplified sequences of an inherited HSR in chromosome 1 of the house mouse (Mus musculus). Mol. Cell. Biol. **11**, 2229–2235.

18. Eckner, R., Ellmeier, W., and Birnstiel, M. L. (1991): Mature mRNA 3'end formation stimulates RNA export from the nucleus. EMBO J. **10**, 3513–3522.

19. Hendlich, M., Lackner, P., Weitckus, S., Floeckner, H., Froschauer, R., Gottsbacher, K., Casari, G., and Sippl, M. (1991): Identification of Native Protein Folds Amongst a Large Number of Incorrect Models. J. Mol. Biol. **216**, 167–180.

20. Hiller, S., Breit, S., Wang, Z.-Q., Wagner, E. F., and Schwab, M. (1991): Localization of regulatory elements controlling human MYCN expression. Oncogene **6**, 969–977.

21. Khazaie, K., Panayotou, G., Aguzzi, A., Samarut, J., Gazzolo, L., and Jurdic, P. (1991): EGF promotes *in vivo* tumorigenic growth of primary chicken embryo fibroblasts expressing v-*myc* and enhances *in vitro* transformation by the v-*erb*A oncogene. Oncogene **6**, 21–28.

22. Kiefer, F., Wagner, E. F., and Keller, G. (1991): Fractionation of mouse bone marrow by adherence separates primitive hematopoietic stem cells from *in vitro* colony-forming cells and CFU–S. Blood **78**, 2577–2582.

23. Lengauer, C., Eckelt, A., Weith, A., Falkenstein, N., Ponelies, N., Miller, K., Lichter, P., Greulich, K. O., and Cremer, Th. (1991): Selective staining of defined chromosomal regions by in situ hybridization of libraries from laser-microdissected chromosome. Cytogenet. Cell Genet. **56**, 27–30.

24. Moll, T., Tebb, G., Surana, T., Robitsch, H., and Nasmyth, K. (1991): The role of phosphorylation and the CDC28 protein kinase in cell cycle regulated nuclear import of the Saccharomyces cerevisiae transcription factor SWI5. Cell **66**, 763–768.

25. Morrison, L. E., Boehmelt, G., Beug, H., and Enrietto, P. (1991): Expression of v-rel in a replication-competent virus: Transformation and biochemical characterization. Oncogene **6**, 1657–1666.

26. Nadeau, J. H., Herrmann, B. G., Bucan, M., Burkart, D., Crosby, J. L., Erhart, M. A., Kosowsky, M., Kraus, J. P., Michiels, F., Schnattinger, A., Tchetgen, M.-B., Varnum, D., Willi-

son, K., Lehrach, H., and Barlow, D. P. (1991): Genetic maps of mouse chromosome 17 including 12 new anonymous loci and 25 anchor loci. Genomics **9**, 78–89.

27. Nasmyth, K. (1991): Cell cycle research proliferates. The New Biologist **3**, 955–958.

28. Nasmyth, K., and Dirick, L. (1991): The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast. Cell **66**, 995–1013.

29. Nicklin, M. J. H., and Casari, G. A. (1991): Single site mutation in a truncated Fos protein allows it to interact with the TRE in vitro. Oncogene **6**, 173–179.

30. Price, C., Nasmyth, K., and Schuster, T. (1991): A general approach to the isolation of cell cycle regulated genes in the budding yeast, Saccharomyces cerevisiae. J. Mol. Biol. **218**, 543–556.

31. Rüden von, T., Mouchiroud, G., Bourett, R. P., Ouazana, R., Blanchet, J.-P., and Wagner, E. F. (1991): Expression of human CSF-1 receptor induces CSF-1 dependent proliferation in murine myeloid but not in T-lymphoid cells. Leukemia **5**, 3–7.

32. Simon, D., Knowles, B., and Weith, A. (1991): Abnormalities of chromosome 1 and loss of heterozygosity on 1p in primary hepatomas. Oncogene **6**, 765–770.

33. Smith, H. O., Tabiti, K., Schaffner, G., Soldati, D., Albrecht, U., and Birnstiel, M. L. (1991): Two-step affinity purification of U7 small nuclear ribonucleoprotein particles using complementary biotinylated 2'-0-methyl oligoribonucleotides. Proc. Natl. Acad. Sci. USA **88**, 9784–9788.

34. Steinhardt, M. (1991): Gentechnologie in der Krebsforschung. CliniCum, May 1991, 24–25.

35. Steinhardt, M. (1991): Erbkrankheiten – eine heikle Frage, CliniCum July/August 1991, 24–26.

36. Steinhardt, M. (1991): Menschen 2. Klasse durch Gendiagnostik? CliniCum October 1991, 22–24.

37. Steinhardt, M. (1991): Schwerpunkte in der Krebsforschung. ÖAZ **45**/31–32, 650–651.

38. Steinhardt, M. (1991): Gendiagnostik – 2 Seiten eines Talers. ÖAZ **45**/44, 960.

39. Steinhardt, M. (1991): Krebs-Lichtblicke, Austria Innovativ **2**, 33.

40. Superti-Furga, G., Berger, G., Picard, D., and Busslinger, M. (1991): Hormone-dependent transcriptional regulation and cellular transformation by Fos-steroid receptor fusion protein. Proc. Natl. Acad. Sci. USA **88**, 5114–5118.

41. Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B. and Nasmyth, K. (1991): The Role of CDC28 and Cyclins during Mitosis in the Budding Yeast S. cerevisiae. Cell **65**, 145–161.

42. Taba, M. R. M., Muroff, I., Lydall, D., Tebb, G., and Nasmyth, K. (1991): Changes in a SWI4, 6-DNA-binding complex occur at the time of HO gene activation in yeast. Gen. & Dev. **5**, 2000–2013.

43. Tönjes, R., Weith, A., Rinchik, E. M., Winking, H., Carnwath, J. W., Kaliner, B., and Paul, D. (1991): Microclones derived from mouse chromosome 7 C-D bands map within the proximal region of the c140CoS deletion in albino mutant mice. Genomics **10**, 686–691.

44. Vennström, B., Beug, H., Damm, K., Engel, D., Gehring, U., Graf, T., Munoz, A., Sap, J., and Zenke, M. (1991): Biological Effects of the v-*erb*A Oncogene in Transformation of Avian Erythroid Cells. In: Molecular Mechanisms in Cellular Growth and Differentiation (Bellve, A. R. and Vogel, J. H., eds). Academic Press, New York and London, pp. 137–147.

45. Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991): Transferrin-polycation DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc. Natl. Acad. Sci. USA **88**, 4255–4259.

46. Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H., and Birnstiel, M. L. (1991): DNA-Binding Transferrin Conjugates as Functional Gene Delivery Agents: Synthesis by Linkage of Polylysine or Ethidium Homodimer to the Transferrin Carbohydrate Moiety. Bioconj. Chemistry **2**, 226–231.

47. Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M. and Noe, Ch. R., (1991): A simple procedure for the preparation of protected 2'-0-methyl or 2'0-ethyl ribonucleoside-3'-0-phosphoramidites. Nucl. Acids Res. **19**, 5965–5971.

48. Wagner, E. F., Wang, Z.-Q., Grigoriadis, A. E., Möhle-Steinlein, U., Aguzzi, A., and Risau, W. (1991): Analysis of oncogene function in ES cells and chimaeric mice. In: Origins of Human Cancer, CSH Lab. Press, 1030–1036.

49. Wagner, E. F. (1991): Transgenic mouse models for bone and vascular diseases and gene transfer into hematopoietic cells. In: Human Gene Transfer (eds. O. Cohen-Haguenauer, M. Boiron) **219**, pp. 227–234.

50. Wang, Z., Grigoriadis, A., Möhle-Steinlein, U., and Wagner, E. F. (1991): A novel target cell for c-fos induced oncogenesis: development of chondrogenic tumors in embryonic stem cell chimaeras. EMBO J. **10**, 2437–2450.

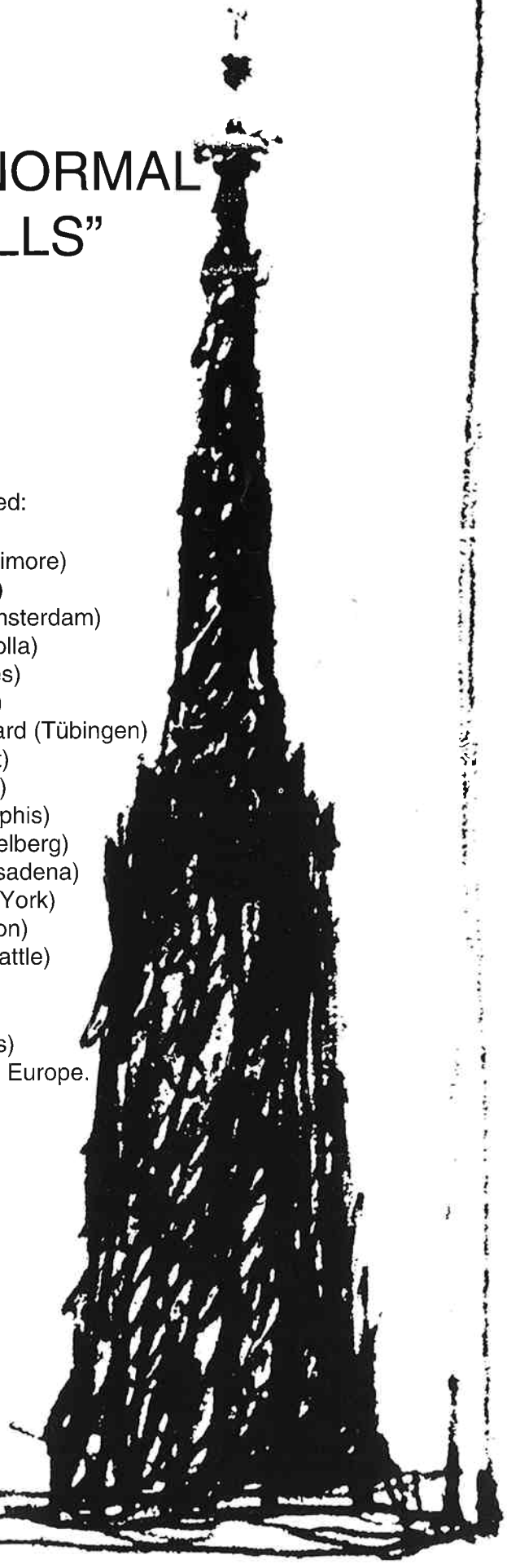
51. Winking, H., Weith, A., Boldyreff, B., Moriwaki, K., Fredga, K., and Traut, W. (1991): Polymorphic HSRs in chromosome 1 of the two semispecies Mus musculus musculus and Mus musculus domesticus have a common origin in the ancestral population. Chromosoma **100**, 147–151.

52. Zenke, M., Khazaie, K., and Beug, H. (1991): V-myc-transformed macrophages expressing the normal human EGF receptor are induced to proliferate by EGF via a non-autocrine mechanism. in Molecular Biology of Haematopiesis. Edited by Sachs, I., Abraham, N. G., Weidemann, C., and Konwalinka, G., pp. 453–467.

53. Zink, D., Weith, A., Martinsson, T., and Schwab, M. (1991): Analysis of chromosome band 1p36 alterations by chromosomal in situ suppression hybridization with a microclone DNA bank. Genes, Chromosomes and Cancer **3**, 407–410.

RESEARCH INSTITUTE OF MOLECULAR PATHOLOGY (I.M.P.), VIENNA
4th INTERNATIONAL CONFERENCE ON

"THE CONTROL OF PROLIFERATION IN NORMAL AND MALIGNANT CELLS"



organised by: Kim Nasmyth

to be held at The Austria Center, Vienna
from 3rd to 5th May 1992

The following invited speakers have already accepted:

P. Bäuerle (Munich)
M. Bishop (San Francisco)
T. Curran (Nutley)
G. Daley (Cambridge USA)
T. Graf (Heidelberg)
E. Harlow (New York)
L. Hartwell (Seattle)
D. Housman (Cambridge USA)
T. Hunt (Cambridge)
S. Jentsch (Tübingen)
J. Kimble (Madison)
D. Koshland Jr. (Berkeley)
W. Lee (La Jolla)
C. Lehner (San Francisco)
A. Levine (Princeton)
R. Losick (Cambridge USA)
S. McKnight (Baltimore)
M. Méchali (Paris)
W. Moolenaar (Amsterdam)
J. Newport (La Jolla)
E. Nigg (Epalinges)
P. Nurse (Oxford)
C. Nüsslein-Volhard (Tübingen)
M. Oren (Rehovot)
S. Reed (La Jolla)
M. Roussel (Memphis)
H. Schaller (Heidelberg)
P. Sternberg (Pasadena)
B. Stillman (New York)
T. Weinert (Tucson)
H. Weintraub (Seattle)

Registration fee: US $ 100,– (US $ 50,– for students)
This fee will be waived for participants from Eastern Europe.

WE CANNOT PROVIDE ANY FINANCIAL ASSISTANCE WITH TRAVEL OR LODGING

For application forms please write to:

Diane Turner, **IMP**
Dr. Bohr-Gasse 7
A-1030 Vienna, Austria
Tel: (222) 79 26 36-654 / Fax: (222) 78 71 53

Registration closes: 31st March 1992