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I.M.P. Scientific Report 1993

Introduction

After the I.M.P. had been in operation for 5 years, the sixth year, 1993, was one of change. Genentech decided to bow out of its part in supporting the I.M.P. while Boehringer decided to take over its share so that in July we became a 100% Boehringer Ingelheim research institution. As stated in the press release and later confirmed by discussions with Prof. Krebs, Deputy Chairman of the Boehringer Board, and Prof. Hohbach, Research Director of Boehringer Ingelheim International (BII), the purpose of the I.M.P. remains to carry out basic research in the area of growth control, differentiation and oncogenesis. The existence of a single sponsor now made it possible to learn explicitly about the research goals of the different branches of the Boehringer Pharmaceutical Industries. A new role for the I.M.P. has emerged in providing advice both to the Max Planck and Bender, subsidiaries of BII, an exercise which the participating Senior Scientists and Group Leaders found both interesting and rewarding.

By the beginning of 1993, all those University departments housed in the building adjoining the I.M.P., and which-together with the I.M.P.-constitute the "Vienna Biocenter", were in place, adding additional scientific weight to the campus at Dr. Bohn-Gasse. We now have a joint library with the University and this has made possible a considerable increase in the number of journals. In order to maintain and augment the international flavor of the I.M.P. at the level of Ph.D. students, the I.M.P. successfully launched an international Ph.D. program, also open to Austrian students, which brought in great many applications from all over the world. Eventually 8 students from a short list of 20 were selected for the program and have since started their work.

Instead of the yearly I.M.P. conference the Director of the I.M.P. and John Gurdon of the University of Cambridge organized a conference for the International Society of Developmental Biologists in August which was open to many Austrians free of charge. At approximately the same time, building work, generously supported by the City of Vienna, started on the sheds abutting the I.M.P. The extension will provide the I.M.P. and the University departments with a very satisfactory library facility in which the back issues of journals from the University departments can be displayed and made available to the scientists of the Biocenter. As of October 1st, Group Leader Thomas Jennewein joined the I.M.P. He will continue his studies initiated at the University of San Francisco on the genetic elements responsible for allowing access of transcription factors to chromatin structures. He replaces Martin Nicklin who departed in 1992 and is now a lecturer at the Section of Molecular Medicine, Royal Hallamshire Hospital, Sheffield, UK.

At the I.M.P.-recess postdocs and Ph.D. students presented 45 talks. There were also presentations from Bender which blended well with those of the I.M.P. In their yearly report the Scientific Advisory Board expressed satisfaction with the progress the Institute had made in the proceeding year and affirmed that the I.M.P. had matured and essentially achieved the goal set out at its inception, namely to establish an international center of excellence for basic research on the molecular basis of disease.

Max L. Birnstiel
Managing Director
Vienna, January 1994
Tumor Vaccines

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Introduction

Higher organisms have developed powerful mechanisms to distinguish self from non-self. Distinction of non-self from self relies on the presentation at the cell surface of peptides derived from cell internal proteins. In most somatic cells this occurs in the MHCII context, or in the case of antigen-presenting cells such as macrophages, dendritic cells, etc., presentation occurs in the MHCII context. These antigens may be recognized by CD8+ cytotoxic and CD4+ T-helper T-lymphocytes, respectively. If a recognition of neoplastic cells, T-helper cells are stimulated and clonal-ly expanded and secrete cytokines which recruit further cells of the immune system, leading to eradication of the cells expressing the foreign antigen. Productive interaction of cytotoxic T-lymphocytes with antigens leads to activation and in turn expansion of the interactive T-cell clones and eventually to the destruction of the cells expressing the foreign antigens.

It is somewhat surprising that cancer cells, all of which are now suspected to harbor mutated oncogenes or tumor suppressor genes, and therefore to express aberrant cellular proteins are not recognized as "foreign" by the immune system, especially since in some cancers - such as melanoma - foreign antigens at the surface of tumor cells have been identified for example, Mage1 is such a protein (van der Bruggen et al., 1991). This leads to the suspicion that foreign antigens are indeed present on most, or at least many, tumor cells and that tumor cells are not rejected as foreign because the distribution and nature of tumor antigens to the presented foreign antigens is inadequate.

If tumor-specific antigens can be identified on all or most of tumors it is conceivable that some day antibodies against the tumor antigens may be used to combat cancer or that recombinant antigens can be used to elicit cellular immunity against tumors. Since to date the distribution and nature of tumor antigens is mainly unknown, attempts are being made to use the entire (cytokine-expressing) tumor cell as a source of antigen and to rely on the immune system to seek out and to mount a response against such foreign antigens. Cytokine-secreting tumor cell preparations have been called operationally "tumor vaccines", a term which we shall also use in this report.

It was found empirically, first by Pardoll and his colleagues in 1990 (Fearon et al., 1990), that immunological tolerance of tumors can be broken by transfusing tumor cells with IL-2 gene expression vectors. When such cells are transplanted into syngeneic mice, a powerful systemic response based on T-lymphocytes is mounted by the organism leading to the destruction of both the IL-2 expressing tumor cells as well as parental tumor cells (not expressing cytokines) injected at distant sites. The systemic response does not derive from an increased systemic level of IL-2 as a consequence of implantation of cytokine producing tumor cells, rather it is the high level of cytokines arising locally, which is thought to have a dramatic effect on reprogramming of immune cells.

This initial observation has led to an avalanche of rather unorganized studies for many mouse tumor models using different cytokine genes (reviewed by Zatloukal et al., 1993) in which rejection of parental tumor cells was reproduced using the above mentioned strategy. It is hoped that such findings will ultimately lead to clinical protocols in which the primary tumor will be removed from patients, the tumor cells set into culture, transfected with cytokine expression vectors, inactivated with X-rays and implanted as tumor vaccines back into patients. If a strong immune response results against these implanted cells one can be hopeful that this procedure will lead to an eradication of distant micrometastases which have arisen previously from disseminated tumor cells.

The utility of transferrinreinfecition in generating tumor vaccines

During the past few years Matt Cotten, while working in the Birnstiel group, and Ernst Wagner, while employed by the I.M.P., as well as David Curiel from the University of North Carolina developed and perfected an adenovirus-augmented, receptor-mediated DNA transfer technique which became known as "transferrinreinfecition" (Curiel et al., 1991; Cotten et al., 1992; Wagner et al., 1992). One of the outstanding features of transferrinreinfecion is that with this technique a multiplicity of DNA plasmids (per cell) can be introduced into a large fraction of cells, both from cell lines and primary cell cultures, including freshly prepared human melanoma cells (see report Ernst Wagner's group). This transfecion leads to extraordinarily high levels of cytokine or reporter gene expression. This seemed a good starting point for the generation of tumor vaccines by the procedure described above and the idea was investigated. Melanoma appeared to be a particularly interesting target for our efforts because there is ample evidence that melanomas are subject to immunological control in humans. For instance, there is a (transient) regression of skin cancers in about 25% of the patients and complete remission in about 0.5%, and a tumor-specific antigen (MAGEI) has been identified (van der Bruggen et al., 1991). In addition cytokotoxic T-lymphocytes directed against the tumor can be obtained from patients.

Melanoma mouse models

Kurt Zatloukal and Manfred Berger

All necessary parameters of cell culture, tumorigenicity and transfection were established for the murine M3 tumor cell line which is derived from a spontaneous melanoma isolated from a DBA mouse in the 1940s. As anticipated from the literature, transfection of M3 cells with an IL-2 expression vector, followed by irradiation and implantation of M3 cells, reduced the tumorigeni-
The more aggressively growing B16-F10 murine melanoma cell, which is syngeneic with C57Bl/6 mice and has the capacity to metastasize was also investigated. This cell is poorly immunogenic by itself, but again systemic immunity can be induced by expression of IL-2 at appropriate levels (see below).

In most experiments carried out and reported in the literature to date, healthy mice which bear no tumor burden are taken for tumor vaccine experiments. The challenge with tumor cells at a distant site is equated with setting up a “micrometastasis” of 10^2 or 3x10^3 tumor cells. This experimental set-up is somewhat artificial and is essentially a prophylactic model where eradication of pre-existing metastases through vaccination would approximate the human condition better. Such therapeutic models (type I) are more cumbersome to carry out and there are at least two reports of this type of experiments. Furthermore, since humans with tumors may be immune-suppressed, the strategy which would mimic the human condition best would be to generate solid tumors known to give rise to metastases in mice, to then remove the primary tumor, treat the animals with tumor vaccine cell preparations and thus demonstrate suppression of the appearance of metastases. We refer to this protocol as representing a type II therapeutic intervention.

For IL-2, expression of about 1,000 to 2,000 units/24h/mouse, a level that we now define as medium, was found to afford almost full protection. Surprisingly, high levels of 5,000 units/24h/mouse or higher levels completely abolished the vaccination effect. In this sample, all animals developed tumors similar to the control groups which received either transfected irradiated cells or cells transfected with an irrelevant DNA. When cells were engineered to produce significantly less IL-2 than the medium level, protection dropped to about 50%, and this level remained at a similar level even when the vaccine production was as little as 5 units/24h/animal. The situation for the B16-F10 tumor cells is under investigation.

While in the prophylactic model vaccination comes before the challenge with viable cancer cells, this order can be reversed in an attempt to obtain a therapeutic model (type II). Here, animals are injected subcutaneously or intravenously (see below) with live tumor cells at a low dose, but a dose still high enough to elicit tumors in all of the non-vaccinated animals. The waiting period of several days to one week without further treatment allows the tumor cells to establish themselves in these animals and to form microscopic tumors. It is at this stage that mice are then vaccinated with irradiated, cytokine-producing tumor cells with a view to eliminate the pre-existing micrometastases. Such experiments were performed with the M3 system. Both vaccines, M3-IRL-2 and low GM-CSF producers, prevented the outgrowth of pre-existing tumors in about 50% of the animals, whereas non-vaccinated mice or animals vaccinated with irradiated cells alone developed tumors in at least 90% of the cases.

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**Prophylactic and therapeutic models**

_Walter Schmidt, Kurt Zatzoukai, Manfred Berger, Elke Herbst, Norma Howells and Cornelia Gabler_

The high efficiency of transfer in infection, where in vitro expression values as high as 200,000 units of IL-2 or 20 μg GM-CSF per 10^6 cells can be reached at 24h, permitted estimation of the effects of cytokine expression on the generation of tumor immunity over many orders of magnitude. In particular, the effects of different cytokines at different expression levels in both M3 and B16 melanoma systems were investigated. In these experiments, healthy mice were vaccinated twice at weekly intervals with 10^5 cytokine-expressing, irradiated tumor cells and were then challenged a week later with 3x10^3 live tumor cells in the case of M3, and 10^4 live tumor cells in the case of B16 cells.

Both vaccines, IL-2- and GM-CSF-releasing M3 tumor cells were found to protect animals in a dose-depndent manner (Fig. 2). In the case of GM-CSF, expression levels below 100 pg/24h/mouse (measured in petri dish cultures in parallel with the mouse experiments) failed to protect the animals from tumor challenge. Protection started around 500 pg/24h/mouse and increased steeply with increasing GM-CSF doses and remained at high level up to 60 ng/24h/mouse, the highest levels studied so far.
Therapeutic experiments with the B16-F10 melanoma cell line which is syngeneic with C57BL/6 mice and is highly metastatic, especially when injected intravenously, were also carried out. 10^6 live B16 cells were injected into the tail-vein of mice. After a waiting period of 3 days allowing establishment of the tumor foci in the lung, mice were vaccinated with irradiated, cytokine-producing B16 cells subcutaneously on day 3 and twice more at weekly intervals. 4 weeks after the initial challenge with live tumor cells animals were examined for metastases. Irradiated B16 cells expressing IL-2, GM-CSF or INF-γ at high, medium and low levels were used as vaccines.

The number of metastases in the lung were scored and the results show that the vaccinated animals were protected in a dose-dependent manner, while B16 cells transfected with an irrelevant DNA or simply irradiated cells gave very high levels of metastases development.

IL-2 medium (3,000 - 4,000 units/24h/mouse) gave the highest protection, with the lungs of almost two thirds of the animals remaining tumor-free. Low expression (300 - 400 U/24h/mouse) protected a quarter of the mice whereas high expression (15,000 U/24h/mouse) failed to immunize. For GM-CSF lesser protection was obtained at all levels. High GM-CSF expression (500 ng/24h/mouse) afforded protection in around 25% of the cases whereas lower expression (≤ 5 ng/24h/mouse) none of the animals were tumor-free. The protection efficiency of IFN-γ was similar to that found for IL-2. These experiments suggest that so far IL-2 appears to be the best cytokine for therapeutic intervention. The failure of protection of the animals after secretion of very high levels of IL-2 in both the prophylactic (see above) and the therapeutic situation is puzzling and reasons for this fall-off in efficiency are now being investigated.

In the future we shall extend our studies to cytokine combinations. An obvious choice would be IL-2 medium plus IFN-γ high (see section on cytokine cascades), since this combination might have synergistic effects.

Conditions for studying the CT26 colon carcinoma model in a similar therapeutic context have been established. Colon cancer being a very frequent tumor in man, these studies may give important leads for the treatment of the human disease.
Fates and effects of cytokine-expressing tumor cells in vivo
Gerhard Maass, Kurt Zaitoukhal, Michael Buschle, Ernst Wagner and Manfred Berger

The competitive PCR amplification technique with an appropriate internal standard for quantitative evaluation was adopted to determine the survival time of IL-2 plasmid and adenoviral DNAs of subcutaneously injected, transferrin- and irradiated (20,000 rad) M3 or B16 cells. The longevity of the DNAs was taken as a measure for the survival of the cell vaccine at the vaccination site. DNAs amenable to PCR remained detectable for up to 2 to 5 days in the case of M3 cells and 10 days for the B16 cells suggesting a rather rapid rejection of these cells.

From these results a schedule of repeated weekly revaccinations appears warranted. The possible transfer of recombiant or viral DNAs to nearby lymph nodes, to different organs of the animals including testes and ovaries, as well as macrophages and monocytes in the blood was investigated at high sensitivity with the result that no such transfer was detectable. This has important bearings for the establishment of the clinical protocol which is being assembled by Prof. Sting.

One tenet of the tumor vaccine approach is that the effects of cytokines are restricted to the immediate vicinity of the inoculum of secreting tumor cells (see above) and no systemic levels of cytokines are obtained. That this seems highly likely was suggested by the finding that at the standard inoculum of 10⁴ secreting cells no elevated systemic blood values could be found for either IL-2 or GM-CSF.

The types of immune cells which may be responsible for rejection of tumors in vaccinated animals were also determined to obtain clues as to how systemic immunity against challenge with parental M3 cells might come about. For this, unmodified tumor inoculum from vaccinated and control animals was investigated by immunohistology of tissue sections taken at different time points after challenge. Cell Markers for CD4⁺, CD8⁺ and Mac-1⁺ cells (Mac-1 recognizes macrophages, natural killer cells and granulocytes) were used. A clear picture emerged: In IL-2 vaccinated animals there was a large influx of macrophages as well as many CD4⁺, but practically no CD8⁺ cells at early times (48h). At a later stage (96h) the inoculum was completely invaded and dispersed by inflammatory cells which again consisted mainly of macrophages; in addition to enormous macrophages, CD4⁺ and CD8⁺ T-cells could be detected in equal proportions. Invasion of macrophages was not as pronounced in control animals immunized with irradiated M3 cells lacking IL-2 expression and practically no T cells were detectable.

In early publications of other authors secretion of IL-2 by tumor cells was seen as circumventing activation of CD8⁺ cells by CD4⁺ helper cells, thus questioning the role of CD4⁺ cells in the process of tumor destruction. By contrast, in our experiments we see an early appearance of CD4⁺ T lymphocytes followed by detection of a larger number of CD8⁺ cells at later time points suggesting a critical role of CD4⁺ cells in rejection of the tumor. In order to explore the importance of CD4⁺-mediated CD8⁺ T cell activation we are currently investigating cellular distribution over time at immunization sites.

Cytokine cascades at the vaccination site
Gerhard Maass

One of the puzzling findings in the tumor vaccine field is that many different kinds of cytokine gene expression vectors can be used for the generation of tumor vaccines, giving similar (but not identical) results. One explanation for this may be that the immune system is integrated by interconnected cytokine cascades. In an attempt to analyze the cascade of secondary cytokines and to understand the steps leading to activation of the immune cells, an investigation of cytokine cascades was initiated at the level of cytokine mRNAs by RT-PCR of the inoculum at the vaccination site.

Commercially available primers for this analysis allows simultaneous detection of mRNAs encoding IL-1α, IL-12, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, GM-CSF, IFN-γ, TNF-α or TGF-β.

Non-transfected M3 cells already produce measurable quantities of IL-6 and TGF-β, B16 cells IL-6, TNF-α and TGF-β and CT26 cells IL-6, IL-7 as well as TGF-β mRNA.

A common finding following the injection of B16-F10 cells into syngeneic mice is the appearance of IL-1 and IL-6. A possible interpretation of this finding is that these kinds of mRNAs may be the signature of macrophages invading and destroying the inoculum (see above). In agreement with their similar action, different transferrin- and cytokine genes elicit secondary cytokines, but there are also some interesting differences. For instance, GM-CSF mRNA becomes detectable when the cell vaccine expresses IL-2. However, the reverse is not true. The GM-CSF expressing cell vaccine reveals no detectable IL-2 RNA. IFN-γ appears to stimulate IL-1 and IL-6 mRNA production particularly well and may therefore provide a strong support for a productive inflammation reaction at the vaccination site.

The study of the cytokine cascade and identification of the cell types involved may eventually provide important leads for the choice of cytokines and combinations thereof for obtaining a maximal vaccination effect.
HISTONE H1A IS AN IN VIVO SUPPRESSOR OF OOCYTE-TYPE 5S GENE TRANSCRIPTION
Harald Kandolf
in collaboration with Eva Dworkin, Bender + Co. Ges.m.b.H., Vienna.

The large family of oocyte-type 5S RNA genes in the frog Xenopus laevis undergo stepwise repression during early development. A possible selective and dominant role of histone H1 in this regulatory process has been suggested on basis of in vitro investigations, but the biological relevance of these observations has not yet to be shown.

My thesis work is the first in vivo demonstration of a biological function for the ubiquitous structural protein histone H1 in the regulation of a specific gene.

The first part of the experimental work concerned a quantitative and qualitative analysis of the somatic H1 variants H1A, H1B, H1C and the maternal variant H1m in early Xenopus embryogenesis. The different histone proteins were identified by means of highly specific polyclonal antiserum raised against each variant using short synthetic peptides as antigens for rabbit immunization. Expression vectors enabled a quantitative "Western" and "Northern" analysis.

Somatic histone H1 was found to be absent from egg and early embryonic chromatin and to be replaced by the abundant maternal variant H1m. The histone H1a stage was characterized by a particularly low histone H1 content. In spite of a high maternally-inherited pool of mRNA, the main somatic variant H1a was hardly detectable during cleavage stage, but accumulated significantly during an up in synthesis at the beginning of this stage. This study represents the first description of a sequential histone expression in the early development of a vertebrate.

The first significant appearance of H1A on embryonic chromatin correlates well with the downregulation of oocyt 5S RNA genes. The aim of the in vivo approach was the selective inhibition of the boost in H1A synthesis at gastrula by a specific knock-out of H1A mRNA. The experimental access was achieved by expression of hamsterhead ribozymes as introns of RNA molecules, a construct which adds the stability characteristics of IRNAs to the specificity of ribozymes. The application in Xenopus embryos led to a selective inhibition of H1A protein synthesis. The lack of histone H1A at gastrula stage prevented oocyt 5S RNA gene expression and even enhanced the expression. This demonstrates that the regulation of this gene family occurs at the chromatin level, histone H1A playing an essential role in this process. This therefore refutes the currently favored model, which explains the differential expression of somatic and oocyt 5S RNA genes on the basis of a stepwise repression of oocyt 5S RNA genes as a consequence of the sequential histone H1 expression in early development. The characteristics of the major oocyte-type 5S gene repeat seem to predispose nucleosomes to H1-mediated positioning on 5S RNA genes and to favour the subsequent formation of an ordered chromatin fiber.

CLEFT STAGE HISTONES: A NOVEL FAMILY OF VERY DISTINCT HISTONES
Birgit Mandl
in collaboration with Meinrad Busslinger, I.M.P.

Cleavage stage (CS) histones are the first histones to appear in the chromatin of the developing sea urchin embryo. They are synthesized during oogenesis and give rise to a maternal store in the egg and also during the first few cell divisions in the cleaving embryo, where they were first identified (Newrock et al., 1979). The CS histones are not only a constituent of the maternal chromatin of the oocyte, but are also used to replace the sperm-specific histones in the male pronucleus immediately after fertilization. This histone exchange dramatically remodels the paternal DNA, as reflected by a decrease in nucleosomal spacing from 250 bp in sperm to 190 bp in the cleaving embryo (reviewed by Poccia, 1989). Chromatin remodeling is then followed by transcriptional activation and replication of the paternal genome.

CS histones have so far resisted all cloning attempts based on screening with heterologous protein probes, suggesting that they differ considerably from other sea urchin histone variants. CS histones were purified from nuclei and post-nucleosomal DNA sequencing of CS histones isolated from Paracentrotus lividus embryos (Brandt et al., personal communica tion) finally led to the isolation of the CS histone cDNA: degenerate PCR primers derived from partial protein sequence data were used to amplify specific cDNA fragments from egg poly(A)+ RNA of the sea urchin. This chromatin elution of an egg cDNA library with these PCR fragments resulted in the isolation of full length cDNA clones for all five CS histones (H1, H2A, H2B, H3 and H4). The cloned cDNAs were identified as cleavage stage histone cDNAs, as their open reading frames encoded the peptide sequences previously obtained by the microsequencing. All five CS genes are encoded by novel genes which are coordinately expressed in the egg and early embryo up to the 128-cell stage. These genes contain introns, possess exceptionally long leader and trailer sequences and code for poly(A)+ RNA due to the presence of a poladenylation signal instead of the 3' terminal palindrom which is characteristic for the replication histone genes. Taken together, these data indicate that the CS histone genes belong to the minor group of replication histone genes which are expressed throughout the cell cycle.

CS H2A and CS H2B proteins are only distantly related to the other H2A and H2B variants of the same sea urchin species, thus indicating that the CS histone genes evolved a long time ago. In agreement with this, the CS H3 and H4 variants also differ from the standard H3 and H4 proteins encoded by other histone genes of the sea urchin. This is a remarkable finding since the core histones H3 and H4 have been exceptionally well conserved in evolution. The CS H1 histone is the most peculiar member of the cleavage stage histone family. It not only represents the longest H1 protein ever described, but it also contains a unique stretch of acidic amino acids at the N-terminus that is usually basic in H1 histones. The core region of the CS H1 protein shows low sequence homology (~40%) with other sea urchin H1 variants, but it shares 60% identity with the core region of H1m, the early embryonic histone H1 protein of Xenopus laevis (Smith et al., 1988). Both proteins, CS H1 and H1m, are significantly bigger than the average histone H1 and both are expressed from replacement variant histone genes. This high degree of conservation suggests that the H1m and CS H1 proteins serve a similar function during early development in these two distantly related species.

Publications during the year

I.M.P. Scientific Report 1993

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Oncogene- and Protooncogene Function in Erythroid Progenitors and Polarized Epithelial Cells

Senior scientist Hartmut Beug
Staff scientist Ernst Reichmann
Postdoc Marièke von Lindern
Postdoc Peter Steinlein
Postdoc Martin Ott (since Sept. 1993)
PhD student Irene Leitner
PhD student Oliver Wessely
Technician Eva Deiner
Technician Liesbeth Boer

Introduction

In 1993, our focus has changed relatively little (for a more detailed description see report 1992). We still concentrate on elucidating the function and possible cooperation of two types of protooncogenes in normal avian erythroid progenitors, nuclear hormone receptors [thyroid hormone receptor α (TRα/c-erbA) and estrogen receptor (ER)], and receptor tyrosine kinases (TGFR/c-erbB), the avian homolog of the epidermal growth factor receptor that causes mammalian TGFβs as a ligand, and c-kit, the receptor for avian stem cell factor). c-erbA and c-erbB are the normal counterparts of two oncogenes (v-erbA and v-erbB) that are contained in the avian erythroblastosis virus. They are responsible for the rapid, fatal erythroleukemia that this retrovirus causes in chicks. We expect that a better understanding of proto-oncogene function will help us to explain in molecular terms how the respective oncogene function and cooperate in leukemogenesis (Oliver Wessely, Marièke von Lindern and H. Beug, in collaboration with Michael J. Hayman, Stonybrook, New York).

In a related set of projects we try to better understand various aspects of normal and abnormal erythroid differentiation by stably expressing genes of interest in erythroid progenitors that can be induced at will to either self-renew or differentiate. These cells are provided by either infecting erythroid progenitors with retroviruses expressing thermosensitive versions of tyrosine kinase oncogenes (ts-V-erbB, ts-V-sea) or by growing normal erythroid progenitors that express c-erbB and are induced to self-renew by appropriate mixes of growth factors and hormones (see report 1992). In presence of an active oncogene or self renewal factors, large amounts of immature progenitors can be grown, which are then induced to synchronously differentiate by inactivating the ts-oncogenes at 42°C by or replacing self-renewal factors with differentiation factors (Epo/Insulin).

The first group of genes introduced are mammalian cytokine receptors (c-kit, erythropoietin(-Epo) receptor, GM-CSF-receptor, IL-3-receptor), since molecularly cloned avian hematopoietic growth factors (cytokines) and their receptors are, as a rule, not yet available. In 1993, we have concentrated on human c-kit, because of the possibility that it acts qualitatively different than c-erbB, and on the IL-3 receptor and the GM-CSF receptor, since it is not even clear whether the respective cytokines exist in birds (Peter Steinlein, Oliver Wessely).

Transcription factors that may have a function in hematopoietic cell differentiation are the second group of genes introduced into differentiating hematopoietic cells. In 1993, we have mainly concentrated on transcription factors of the Ets-family (ets-1, flt-1) as well as on the p53 tumor suppressor gene. Since these genes...
are involved in two of the three known genetic changes in Friend erythroleukemia, we attempt to elucidate how these genes would contribute to the leukemic phenotypes and how they may cooperate (Maireke von Lindern and H. Beug, in collaboration with J. Ghysdael, Paris). Due to lack of hands (Georg Mellitzer decided to concentrate on projects of the Zenke group), the intended studies on the function of other conditional transcription factors (myc,ER, jun,ER, fos,ER, see report 1992) had to be postponed. We have, however, also focussed on the v-ski oncogene (derived from a transcription factor with weak homology to myc and MyoD and known to affect muscle differentiation) since this oncogene profoundly affects differentiation of erythroid, myeloid and perhaps multipotent cells. It combined with exogenous (ts-v-sea) or endogenous (c-kit) receptor tyrosine kinases (H. Beug, in collaboration with the group of M.J. Hayman, Stonybrook).

The second major research interest of the lab is how oncogenes alter key features of epithelial cells during carcinogenesis, using again a conditional oncogene approach. We employ mammary epithelial cell lines which retain many features of primary mammary gland cells, the most important one being epithelial polarization, i.e. the ability of epithelial cells to express different proteins in different domains (apical or basolateral) of the plasma membrane or the cytoplasm. Cells of such lines are infected with various conditional oncogenes, checked for normal behavior with oncogene function turned off and then analyzed for phenotypic and gene expression changes after turning on oncogene function. In 1993, we have concentrated on a hormone-regulated c- jun-steroid receptor fusion protein (c-jun ER), which seems to affect epithelial polarization in a completely reversible fashion (Irene Leitner, Ernst Reichmann). In addition, we have started to analyze genes acting upstream of c-Jun/c-Fos. Here, we have largely concentrated on the Ha-ras oncogene because of its starting ability to trigger conversion of nonmalignant epithelial cells into those causing tumors in animals, a process that seems to occur both in vivo and in vitro (Ernst Reichmann, Martin Off).

A. Proto-oncogene function in normal erythroid progenitors

Role of the c-erbB/TGF-α-R and c-kit/SCF-R protooncogenes in regulating self renewal and differentiation of erythroid progenitors

In last year’s report, we described two types of normal erythroid progenitors that can be grown from normal chicken bone marrow in presence of specific growth factors and steroid hormones. The first cell type (SCF/ TGFαR progenitors) expresses c-erbB/TGFαR, c-kit and estradiol receptor (ER), is present in chick bone marrow at very low numbers (1 in 15,000) and is induced to sustain self renewal by a ligand for avian c-erbB (TGFαR) plus estradiol. The second, much more frequent progenitor type (SCF progenitors, 1 in 500) expresses only c-kit and is induced to transient self renewal by avian stem cell factor (SCF, Anderson et al., 1990). A second major difference between these two progenitors was the fact that self renewal factors were dominant over differentiation factors (Epo plus insulin) in SCF/TGFαR progenitors, while SCF progenitors differentiated in presence of Epo plus insulin even when SCF was present.

In 1993, we have characterized both progenitor types in more detail. We have also tried to solve the related question, if the two progenitor types are stable, independent cell types or whether one may be the progenitor of the other. One prerequisite for these studies was access to large amounts of avian stem cell factor. Construction of a bioactive SCF derivative tagged with 6 histidine residues and development of a fast affinity purification procedure solved this problem, allowing us to easily produce milligram amounts of pure factor (Peter Bartunek, Peter Steinlein).

Diffenentiation kinetics

Hartmut Beug, Liesbeth Boer in collaboration with Peter Bartunek (group Zenke)

Since the main problem in using SCF progenitors for differentiation experiments was their limited time of self renewal (7-10 days with an increasing tendency to spontaneously differentiate), we first developed purification methods allowing to produce large quantities of 3-4 day old SCF progenitors. A combination of density purification steps plus the use of myeloid growth factors that could not be applied without further purification. These normal progenitors are thus clearly superior to cell systems using conditional oncogenes (ts-v-sea, EGF/α-tv-sea, myb, see prior reports and Knight et al., 1988, Beug et al., 1990), since the latter are much more limited in the quantities of differentiated cells one can produce. Differentiate with less synchrony and are far more variable with respect to the number of cell divisions during maturation.

Gene expression during differentiation

Petr Bartunek (group Zenke) and Hartmut Beug

Because of these obvious advantages and the fact that SCF progenitors behaved exactly as expected for normal CFU-E (colony forming unit erythroid) progenitors, we looked at the expression of growth factor- and hormone receptors, erythroid specific transcription factors and erythrocyte proteins at various times after differentiation induction. Essentially no changes in gene expression (mRNA steady state levels) were observed during the first 12 to 15 hours. Thereafter, the gene expression program underwent major, rapid changes. Gene expression typical for self renewing cells (c-myb, c-kit, m-myb, ER, and - in SCF/TGFα-R progenitors - c-erbB) were rapidly downregulated between 16 and 24 hours after differentiation induction. At the same time, erythroid transcription factors (GATA-1, GATA-2, SCL, NF-E2) were strongly upregulated. Only slightly later (24 hours)

Developmental origin of SCF- and SCF/TGFα-R-progenitors

Hartmut Beug and Peter Steinlein, with M.J. Hayman, New York

Our finding that two types of erythroid progenitors exist in normal bone marrow differing in apparent frequency, self renewal ability and receptor tyrosine kinase expression immediately raised the question, if and how these cells were related. The fact, that the SCF/TGFαR progenitors were so much less frequent than SCF progenitors initially suggested, that the former were the precursors of the latter. According to this idea, SCF/ TGFα-R progenitors would correspond to BFU-E (burst forming unit erythroid) and thus undergo several cell divisions while maturing to the CFU-E stage (corresponding to SCF progenitors). If this were correct, SCF/TGFα-R progenitors should require much more time and should go through significantly more cell divisions before terminal differentiation than SCF progenitors. A detailed analysis of the differentiation kinetics of both progenitor types showed, that SCF- and SCF/TGFαR progenitors differentiated with essentially similar speed (Figure 1). The only possible exception was that some populations of SCF/TGFαR progenitors required a longer time before they shortened their cell cycle and altered their gene expression program. Apart from that, both cell types went through the same number of cell divisions and exhibited the same differentiation kinetics with respect to nuclear condensation, cell shape changes, hemoglobin accumulation, entry into the postmitotic stage and loss of c-kit-erbB and ER expression. These results are incompatible with the above idea that SCF/TGFα-R progenitors are the precursors for SCF progenitors. Rather, they suggest that both progenitors are generated through parallel, but independent pathways.

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Tyrosine kinase inhibitors (tyrophostins): Tools to dissect the signal transduction pathways of c-Kit and c-ErbB

Mariève von Lindern, in collaboration with A. Levitzki, Rehovot, Israel

In 1992, we reported that two tyrosine kinase inhibitors (tyrophostins) specifically inhibited TGFα-induced self renewal in SCF/TGFα-progenitors, while they only marginally affected erythroid differentiation induced by Epo plus insulin in these cells. Since tyrosine kinases are also involved in the Epo- and insulin receptor signaling pathways, these results suggested, that the tyrophostins are specifically suppressing signaling by the endogenous c-ErbB.

One possible way tyrophostins might act is by inhibiting the binding of SH2 domain-containing proteins of signaling pathways to recognition sequences around autophosphorylated tyrosines, which are located in the intracellular, regulatory domains of active receptor tyrosine kinases. Since different receptors bind different patterns of SH2 domain proteins, it is conceivable that tyrophostins could be found that would be specifically inhibiting c-Kit without affecting c-ErbB and vice versa. In orienting experiments, one compound has been found that inhibits c-Kit-induced self renewal in SCF progenitors much better than TGFα-induced self renewal in SCF/TGFα-progenitors. In contrast, some of the compounds active on c-ErbB were only weakly active on c-Kit. All of these compounds left Epo/insulin induced differentiation unaffected. These studies are continued to generate agents eventually enabling us to selectively suppress certain functions of either c-ErbB or c-Kit.

How does the estrogen receptor regulate self renewal of chicken erythroid progenitors?

Mariève von Lindern, Liesbeth Boer and Hartmut Beug, in cooperation with M. Parker, ICRF, London

In 1992, we described that SCF/TGFα progenitors express the estrogen receptor (ER) and require estradiol in addition to TGFα for sustained self renewal. By overexpressing the exogenous human ER in erythroblasts devoid of endogenous ER, we showed that the ER cooperated with c-ErbB/TGFαR in maintaining self renewal and arresting erythroid differentiation. Further analysis showed that the two receptors seemed to cooperate by downstream regulation of distinct, but overlapping sets of erythocyte-specific genes. Furthermore, suppression of ER function by antagonist efficiently inhibited erythroid differentiation by tyrosine kinase oncogenes. These results led to the current working hypothesis that cooperation of c-ErbB/TGFαR and ER in regulating normal progenitor self renewal is comparable to oncogene cooperation between c-ErbB and v-ErbA in AEF-induced acute erythroleukaemia. It was therefore desirable to identify genes, that are directly regulated by the ER in a hormone-dependent fashion.

First, we asked whether the repression of erythocyte genes by the ER was just a consequence of the differentiation arrest it induced or whether the ER (as shown previously for v-erbA) could repress erythocyte genes also in partially mature erythroid cells in which these genes are already expressed at maximum level. Erythroblasts expressing either the exogenous human ER (huER-HD3) or the endogenous chicken ER (SCF/ TGFα progenitors) were induced to differentiate in the presence of the estradiol antagonist ICI 164384, the partially mature cells purified by density gradient centrifugation and then allowed to further mature in presence of estradiol or, as a control, of antagonist. Similar to v-ErbA, the activated ER arrested further maturation of the predifferentiated cells, while the antagonist-treated cells differentiated normally. This demonstrates that the ER not only prevents entry into cell differentiation, but can block differentiation also at later stages (Figure 3).

We then assayed gene expression in the same, partially mature cells prevented from terminal differentiation by estradiol. Since we were interested in rapid changes (2-24 hours), gene expression had to be analyzed by run-on analysis. While a number of erythocyte-specific genes (β-globin, GATA-1, NF-ES, SCL) were transcribed at reduced levels after 8 to 24 hours of estradiol treatment, none of them was affected rapidly (i.e. within 2-1/2 hours). As already seen earlier, the CALL gene seemed to be induced by estradiol. These results made it unlikely that the ER repressed erythocyte genes by direct interaction with estradiol response elements (ERE) in the promoters of erythocyte-specific genes. Furthermore the observed pattern of genes affected by the ER did not really correspond to that repressed by v-ErbA.

Currently we are trying to circumvent these difficulties by analyzing which domains of the estrogen receptor are important for repression of erythroid differentiation and altered regulation of erythocyte-specific genes.

Interaction of tyrosine kinase oncogenes with purified erythroid target cells

Hartmut Beug, Oliver Wessely, Eva Deiner and Liesbeth Boer, in collaboration with M.J. Hayman, New York

In 1992, we found that SCF/TGFα progenitors were rapidly and efficiently transformed by retroviruses expressing tyrosine kinase oncogenes, suggesting that these progenitors represented target cells for transformation by these oncogenes (Graf et al., 1981). These studies have been completed, showing that >50% of the cells in a clonal or nonclonal population of SCF/ TGFα progenitors are actually transformed by retroviruses expressing ts-v-sea, v-erbB or c-erbB. In contrast to the normal progenitors, the v-erbB- or ts-v-sea-transformed cells grow independent of exogenous growth factors, show an altered pattern of cell surface antigens and are capable of spontaneous differentiation in absence of differentiation hormones (Epo plus insulin). Also, many clones lose expression of endogenous receptor tyrosine kinases (c-erbB, c-kit). This receptor loss, however, is not a direct consequence of oncogene expression, since clones coexpressing endogenous c-erbB and ts-v-sea can be isolated. In these clones, the exogenous oncogene and the endogenous c-erbB seem to function independently of each other.

Interestingly, SCF/TGFα progenitors transformed by the c-erbB/TGFαR expressing retrovirus differed from both unfected and ts-v-sea or v-erbB transformed cells. These cells, showing a 20- to 50-fold overexpression of c-erbB/TGFαR, require much higher doses of TGFα in order to self renew. Differentiation in absence of TGFα is delayed and probably disturbed with respect to cell cycle regulation. In addition, hemoglobin accumulation in the developing erythrocytes is impaired. This suggests that overexpression may be sufficient to activate c-erbB to some extent. Furthermore, the cells are unable to spontaneously differentiate in absence of differentiation factors, a property also exhibited by erythroblasts transformed by human c-erbB (Kihara et al., 1988).

A similar series of experiments was done with SCF progenitors. Again, we could show that 10-30% of purified SCF progenitors capable of forming a CFU-E erythroid colony could be transformed by v-erbB, c-erbB or ts-v-sea. The transformed clones resemble those obtained from SCF/TGFα progenitors, but may have a shorter lifespan and somewhat different hormone requirements during differentiation, issues that are currently under study.

We also tried to infect SCF and SCF/TGFα progenitors with oncogenes known to be unable of transforming...
erythroblasts. With the v-myc and v-rel oncogenes, it was impossible to obtain clones expressing the respective oncogenes. Preliminary evidence suggests that v-myc induces apoptosis, while v-rel-expression seems to be specifically suppressed in erythroid cells at both the RNA- and protein-level (G. Meltzter, diploma thesis). In case of v-myb, expressing clones were obtained, but these were still completely dependent on exogenous TGFα plus estradiol and could therefore not be regarded as transformed. We conclude that target cell specificity of tyrosine kinase oncogenes for the erythroid lineage is probably explained by the fact that the oncogenes functionally replace the self-renewal inducing activity of endogenous c-erdb/ TGFα-R, which seems to be specifically expressed in a subset of erythroid progenitors, but not in progenitors from other lineages.

B. Normal and abnormal erythropoiesis: Influence of retrovirus-transduced cytokine receptors and transcription factor oncogenes

Biological activity of three mammalian cytokine receptors (mu EpoR, hu II-3R and hu GM-CSFR) in avian erythroid and myeloid cells

Oliver Wessely, Eva Deiner and Peter Steinbe

In 1992, we demonstrated that retrovirus-transduced mammalian cytokine receptors can be expressed in avian hematopoietic cells and seem to be functional in these heterologous cell types. The reasoning behind this approach was that most mammalian cytokine receptors are not function in avian cells, while avian cytokines and their receptors are, as a rule, not cloned (except CMGF, a possible avian homolog to mammalian II-6, and avian SCF) or even completely unknown.

Murine Epo-Receptor (mu EpoR)

In 1992, we completed the analysis of mu EpoR action in erythroblasts transformed by conditional oncogenes (ts-v-sea, human c-erdb/EGFFR). We could show that the mu EpoR functioned in chicken cells in a way indistinguishable from the endogenous EpoR in mammalian erythroid cells. We then concentrated on expressing the EpoR in normal SCF- or SCF/TGFα progenitors. These experiments, however, failed completely. Although clones could be obtained that responded to hu-r-Epo, they could not be grown up beyond 10^3-10^4 cells.

A possible explanation for this failure was suggested by experiments designed to look at Epo-induced gene expression changes in ts-v-sea transformed erythroblasts expressing the mu EpoR at the permissive temperature (36°C). Although proliferating, these cells strongly upregulated erythroid-specific genes (i.e. β-globin, band 3, GATA-1) while the expression of genes typical for self-renewing cells (c-myc, estrogen receptor) was shut off or heavily reduced. This suggested that the mu EpoR altered the gene expression program of the normal avian progenitors in a fashion incompatible with self-renewal. To overcome this problem, the EpoR will be combined with those genes it may downregulate (i.e. c-myc, c-erdb/TGFα-R) or with suitable oncogenes (v-erbB, c-ets-1, p53; see below).

Human Interleukin 3-Receptor (hu II-3R)

In 1992, we described approaches to introduce both chains of the II-3R (II-3R x chain plus common 8 chain shared with other cytokine receptors like GM-CSFR; see below) into ts-v-sea transformed erythroblasts. Although our studies were severely hampered by the fact that II-3R expressing ts-v-sea erythroblasts had an unusually short lifespan and could not be thawed after freezing in liquid nitrogen, some information about their phenotype was obtained. In essence, the II-3R did strongly enhance the rate of proliferation of cells at the permissive temperature. At the nonpermissive temperature, it again stimulated proliferation, leading to a delay in differentiation and a much higher yield of mature cells. However, it did not seem to cause an arrest in differentiation.

To overcome the problems with the erythroblasts, we have started to express both II-3R-receptor chains in erythroid and myeloid cells transformed by the ts-E26 virus. This virus expresses a fusion protein encoded by the v-myc and v-ets oncogenes plus viral gag sequences and transforms bi- or multipotent progenitors in addition to early erythroid and myeloid progenitors. We expect these cells to be ideally suited to analyze the function of the II-3R since II-3 in the mouse mostly acts on early, multipotent progenitors.

A. hGM-R/ts-sea erythroblasts

B. 3H-thymidine incorporation

C. hemoglobin accumulation

Fig. 4: The mammalian GM-CSF receptor enhances proliferation, but abolishes responsiveness to erythropoietin in differentiating avian erythroblasts.

A. Schematic outline of the experiment. Erythroblasts transformed by the ts-v-sea oncoprotein and expressing the human GM-CSF receptor (hGM-R) or (II-3R) chains (left: solid and hatched receptor symbols) are induced to differentiate at 22°C in the presence of Epo (dotted receptor symbol with black square). Their phenotype is compared to control ts-v-sea erythroblasts expressing no exogenous receptors (hGM-R).

B. The cells described in A were analyzed for thymidine incorporation at day 1 to monitor for differentiation-related proliferation (top) and for differentiation by measuring hemoglobin accumulation at day 2 (bottom). Cells are incubated without factor (dotted bars), with Epo plus insulin (hatched bars) and with recombinant GM-CSF (black bars). As expected, control ts-v-sea cells respond to Epo by both enhanced proliferation at day 1 and hemoglobin accumulation at day 2, while they are unresponsive to GM-CSF. In contrast, GM-CSF expressing ts-v-sea cells are strongly stimulated to proliferate by GM-CSF (usually better than by Epo) but completely fail to accumulate hemoglobin. This inhibition is not dependent on GM-CSF, since it occurs above the presence of Epo, suggesting that the GM-CSFR constitutively abolishes the ability of the cells to undergo differentiation in response to Epo.
Human granulocyte macrophage colony stimulating factor receptor (GM-CSFR).

Last year, we made the unexpected finding that ts-s v-see erythroblasts expressing the human GM-CSFR α chain alone underwent apoptosis as if withdrawn from differentiation factors when shifted to 42°C in presence of Epo/Insulin. Apparently, GM-CSFR a rendered ts- oncogene transformed erythroblasts unable to mount a response to Epo (see report 1992). We have now repeated these experiments with ts v-see erythroblasts expressing the GM-CSFR α chain plus the β chain common to the II-3R and the GM-CSFR. At the permissive temperature, the cells show a strong proliferation response to hu-β-GM-CSF, indicating that the complete receptor was functional in avian erythroid cells. When shifted to the nonpermissive temperature, the GM-CSFR expressing cells completely failed to accumulate hemoglobin, but disintegrated after 1-2 days as immature, apoptotic cells, thus confirming our earlier results. A possible explanation would be that the overexpressed GM-CSFR (~50,000 α chains per cell) scavenges complexing chains or components of the signal transduction machinery also required by the Epo receptor.

Having shown that the complete GM-CSFR is fully active in avian cells, we are currently testing its activity in ts-E26 transformed erythroid, myeloid and multipotent cells, these being the most relevant ones with respect to the known activity of GM-CSF in mammalian hematopoietic progenitors.

Transcription factor oncogenes in leukemia: model studies in the avian system

Reconstructing the Friend-disease in avian erythroblasts

Hartmut Beug and Marieke von Lindern, with J. Gysdael, Paris

During development of mouse erythroleukemia caused by the Friend erythroleukemia virus, the Friend virus glycoprotein gp55 constitutively activates the Epo-receptor, leading to excessive erythropoiesis and giant spleens in the infected mice. When leukemia progresses, development of immature, leukemic erythroblasts is correlated with aberrant overexpression of two transcription factors of the eto-family, spli and flt-1, induced by retroviral insertion into the promoters of these genes. In addition, the reactive oncogene p53 becomes either deleted or mutated during the development of the disease. In an attempt to determine how the single oncogenes contribute to the leukemic phenotype, we have expressed spli, flt-1 and a ts-mutant of p53 in normal and ts v-see transformed progenitors. The emerging result is, that both spli and p53 partially arrest differentiation in erythroid cells.

(i) spli: When introduced into ts-v-see transformed cells, spli clearly retards differentiation after shift to 42°C. The cultures become rapidly heterogenous, consisting of cells at essentially all maturation stages including a significant proportion of immature cells (20-40%) at times when the control cultures have differentiation completely. Differentiation, however, was not completely arrested, because all cells either differentiated or underwent apoptosis after 5-6 days at 42°C.

Interestingly, the differentiation-arresting ability of spli is strongly enhanced by SCF. In normal erythroid SCF- or SCF/FGFα progenitors, spli seemed to cause a similar delayed or aberrant differentiation, but was unable to induce proliferation of these cells or render them independent of exogenous growth factors.

One possibility for the weak biological activity of spli-1 were the low amounts of spli-1 protein expressed in the avian cells. We have therefore constructed a gag-spli-1 fusion gene and inserted it into a ts-v-see expressing retrovirus vector, which is currently tested for biological activity.

(ii) ts-p53. Last year we described the successful construction of an avian retrovirus expressing a temperature sensitive mutant of mammalian p53. Like in mammalian cells, this virus transformed avian fibroblasts at the nonpermissive temperature (39.5°C) while it arrested the fibroblasts at 31°C at the permissive temperature (34.5°C). This virus was then used to infect ts v-see transformed cells. In these, the ts-p53 phenotype was minor. After shift to 42°C, the ts-p53 expressing cells differentiated, but in a delayed and aberrant fashion, yielding abnormally shaped erythrocytes and disintegrated cells. As expected, cell proliferation was arrested at 34.5°C.

We are currently trying to infect normal erythroid progenitors (SCF/FGFα progenitors) with the ts-p53 virus to determine, whether p53 may have a growth promoting effect or may render the cells independent of exogenous growth factors.

The v-ski oncogene cooperates with c-Kit to transform normal chick bone marrow cells

Hartmut Beug and Peter Steinlein, with J. Larsen, S. Meyer and M.J. Hayman, New York

In 1992, we described the biological effects of a retrovirus coexpressing the ts-s v-see oncogene with the avian retroviral oncogene v-ski, a mutated transcription factor involved in muscle differentiation. Surprisingly, v-ski rendered the erythroid differentiation program severely aberrant, combining enhanced expression of some genes (β-globin, band 3) with the repression of others. Since the same virus caused a mixed erythro/myeloid leukemia in chicks and caused the outgrowth of a fibrosarcoma of either erythroid or myeloid transformed clones in addition to erythroid ones, we reinvigated the question whether v-ski alone may be able to transform bone marrow cells exhibiting special growth factor requirements. When bone marrow cells were cocultivated with high titer v-ski retrovirus producing fibroblasts in presence of avian stem cell factor (SCF), proliferating cells could be grown out that expressed the ski oncogene and required SCF and possibly estradiol for proliferation. These cells turned out to be a mixture of immature or mature macrophages, neutrophils, mast-cell like cells and erythroid cells. This raises the possibility that v-ski transforms cells of several lineages or even a multipotent progenitor. Studies are in progress to clarify this issue.

C. Oncogene function in normal epithelial cell differentiation and carcinoegenesis

A conditional c-JunER fusion protein causes estradiol-induced loss of epithelial polarity, which is fully reversible

Irene Leitner and Ernst Reichmann, with Meinrad Busslinger and H. Schwarz, Tübingen

In last year’s report, we described the phenotypes induced in polarized mammary epithelial cells by two oncogene-estrogen receptor fusion proteins (c-FosER, c-MycER). While c-MycER caused hyperproliferation and did not affect epithelial polarity, c-FosER had a dual activity. When activated with estradiol for short time periods (30 min to 2 hours) epithelial polarity broke down in a reversibly fashion, but no changes in gene expression were observed. Long-term activation of c-FosER (>24 hours) caused irreversible loss of epithelial polarity combined with downregulation of epithelial-specific, while certain markers of mesenchymal cells were upregulated at the same time.

In 1993, we have concentrated on c-jun, the partner of c-fos in the AP-1 transcription factor. Our initial approach of infecting mammary gland progenitors able to differentiate into epithelial and myoepithelial cells (see report 1992) had to be postponed, since these cells showed a marked response to estradiol in absence of exogenous oncogenes. c-JunER was therefore expressed in stable, fully polarized epithelial cells, with the following results: in cells seeded onto porous supports (filters) estradiol activated c-JunER prevented building up of a polarized epithelial monolayer, instead the cells maintained wide intercellular spaces and tended to form multilayers (Figure 5A). In the presence of estradiol, the transplasmelar resistance of such cultures remained low, while control cultures treated with antagonist (ICI 164384) formed normal epithelial monolayers with high transepithelial resistance. In already polarized monolayers, estradiol caused the breakdown of epithelial polarity, while depolarized monolayers obtained in presence of estradiol gained normal polarity and high transepithelial resistance when estradiol was replaced by ICI 164364.

We concluded that c-JunER-induced breakdown of polarity was reversible regardless of how long the oncogene had been activated. This finding, which was in stark contrast to the c-FosER results (see report 1992), could be fully confirmed by analysis of polarization markers, i.e. apically and basolaterally sorted proteins. In presence of estradiol, the apical marker Muc-1 and the basolateral proteins uvomorlin (E-cadherin) and desmoplakin became redistributed to the entire cell surface, but were restored to their proper positions after removal of estradiol. In addition, the assembly of cytoskeleton filaments and cytoskeleton tures formed by cytoketlon and β-actin was drastically altered. Interestingly, some proteins involved in the function of junctional complexes (e.g. β-catenin, ZO-1) became essentially undetectable by immunofluorescence in the estradiol-treated cells (Figure 5B). Protein analysis showed, however, that the expression level of β-catenin was not affected by estradiol treatment. It is
therefore possible, that c-Jun prevents the assembly of junctional protein complexes from individual, cytoplasmically located components. This idea is in line with our finding, that estradiol induced no detectable changes in the expression of epithelial or mesenchymal marker genes in the c-JunER expressing cells.

**JunER - mammary epithelial cells**

![Image of experimental setup]

**The Ha-ras oncogene facilitates epithelial-mesenchymal transition in mammary epithelial cells: implications for tumorigenesis**

Ernst Reichmann, Martin Oft and Eva Deiner

Last years finding that long-term activated c-FosER induced a major reprogramming of gene expression in mammary epithelial cells closely resembling the process of epithelial-mesenchymal transition (Hay, 1990). We were therefore interested if proteins located upstream of transcription factors like Jun and Fos in the signal transduction cascade would be able to trigger similar events. In 1993, we have focused on the Ha-ras oncogene, since the Ras proteins play a key role in transmitting signals from receptor tyrosine kinases and other growth factor receptors to protein kinases (MAP-kinase, erk-proteins) that activate transcription factors (c-Jun, c-Ets) by phosphorylation. In epithelial cells (MDCK), Ha-ras was known to cause multilayering and a partial loss of epithelial polarity (redistribution of apical but not basolateral markers), if the cells were seeded on suboptimal extracellular matrix layers. When Ha-ras was introduced into stable, fully polarized mammary epithelial cells, the same phenotype was observed. Injection of these cells into nude mice caused the outgrowth of large tumors, which were of mesenchymal appearance, but expressed low levels of cytokeratins and could be shown to be of donor origin. These results led to the speculation that Ha-Ras facilitated epithelial-mesenchymal transition in response to external stimuli and that this ability may be related to the tumorigenic potential of this oncogene. We have begun to study these questions both in vitro and in vivo. If Ha-ras-expressing epithelial cells were seeded into collagen gels, they initially formed poorly organized clumps under conditions, where control cells form chord- or duct-like structures of polarized epithelial cells. Later mesenchymal cells grew out from these clumps that freely invaded the collagen gel and fully overgrew the few remaining epithelial cells. Analysis of these mesenchymal cells revealed that their expression of epithelial and mesenchymal markers was very similar to both cells induced by long term activated c-FosER (which were, however, completely unable to form tumors) and to the Ha-ras-tumor cells. These results suggested, that the conditions in the collagen gel (whole cell surface exposed to extracellular matrix, serum factors) may trigger epithelial-mesenchymal transition in the Ha-ras expressing cells.

Next, we analyzed whether similar processes could be demonstrated in vivo. For this, small pellets (~ 10^6 cells) derived from Ha-ras-expressing or control mammary epithelial cells were implanted subcutaneously into nude mice and their development studied by histological and histochemical analysis as well as in situ hybridization at various times after implantation. The normal cells, which retained epithelial cell morphology and vimentin expression, did not proliferate and remained as tight clumps. After 4-6 days, the cells in the implant became necrotic, which was then encapsulated by a layer of proliferating, mesenchymal cells of host origin. In contrast, the Ha-ras-expressing epithelial cells neither became necrotic nor induced encapsulation. Rather, mesenchymal cells which were vimentin negative, vimentin positive and weakly positive for cytokeratin started to grow out from the implant around day 3-4, leaving only a few remaining epithelial...
cells (vimentin negative and strongly uvomorulin and cytokeratin positive) at day 8. The mesenchymal cells were of donor origin (as shown by their Ha-Ras expression) and highly proliferating. In later stages, vascularization of the tumor could be observed, a phenomenon that is currently under study. In summary, the Ha-Ras oncogene seems to trigger epithelial-mesenchymal transition when expressed in epithelial cells, that are prevented by the environment from building up polarized multilayered structures (Figure 6). The results are in favor with the assumption that although epithelial-mesenchymal transitions are not by themselves led to carcinomas, they may represent an important step in this process.

Publications during the year


Lee, E.B., Beug, H. and Hayman, M.J. (1993). Muta-
tional analysis of the role of the carboxy-terminal region of the v-erbB protein in erythroid cell transformation. Oncogene 8, 1317-1327.


Other references


Lee, E.B., Beug, H. and Hayman, M.J. (1993). Muta-
tional analysis of the role of the carboxy-terminal region of the v-erbB protein in erythroid cell transformation. Oncogene 8, 1317-1327.


Introduction

The highly conserved paired box gene is characteristic of a small family of developmental control genes that were first described in Drosophila and subsequently in vertebrates (reviewed by Gruss and Wallner, 1992; Noll, 1993). This protein domain of 128 amino acids specifies a novel DNA-binding function and is encoded by nine paired box genes, Pax-1 to Pax-9, which have been isolated from the human and mouse genomes. During embryogenesis these genes are expressed in specific regions of the developing central nervous system (CNS), with the exception of Pax-7 which is expressed in the developing vertebral column. Mutations in Pax-1, Pax-3 and Pax-6 have been associated with the mouse developmental mutants undulata, Splish and Smal, respectively. Moreover, genetic lesions in the human PAN-3 and Pax-6 genes cause Waardenburg's syndrome and aniridia. This evidence implicates Pax proteins as important regulators of mammalian development (reviewed by Gruss and Wallner, 1992).
Therefore constitute a subclass within the Pax family. During the past year we have studied the in vivo function of Pax-5 by targeted mutation in the mouse genome and have continued the structure-function analysis of BSAP (Pax-5) to gain insight into how this transcription factor regulates gene expression. In addition, we have subjected the Pax-5 gene to a detailed expression analysis during mouse ontogeny.

**DNA sequence recognition by Pax proteins: Bipartite structure of the paired domain and its binding site.**

*Thomas Czerny*

The paired domain has recently been identified as a novel DNA-binding motif. Little is, however, known about how the paired domain recognizes DNA. Furthermore, comparison of binding sequences alone was not informative enough to define a reliable consensus recognition sequence for the paired domain, thus suggesting that Pax proteins recognize degenerate DNA sequences.

Previous DNA-binding studies with the B-cell-specific transcription factor BSAP (Pax-5) indicated that an intact paired domain is required for interaction of BSAP with DNA (Adams et al., 1992). We have now identified a subset of BSAP recognition sequences which also bind to a truncated BSAP peptide lacking 16 C-terminal amino acids of the paired domain. Sequence comparison of this class of BSAP-binding sites made it possible to unequivocally align all known BSAP-binding sites and to deduce a consensus sequence consisting of two distinct half sites. We propose a model for the paired domain-DNA interaction in which the paired domain is composed of two subdomains that bind to the two half sites in adjacent major grooves on the same side of the DNA helix (Fig. 1). The existence of these half sites and of the two paired domain subregions was directly demonstrated by methylation interference analysis and by in vitro methylation of both the paired domain and its recognition sequence. Both half sites contribute to the overall affinity of a given BSAP-binding site according to their match with the consensus sequence. However, none of the naturally occurring BSAP-binding sites completely conform to the consensus sequence. Instead, they contain compensatory base changes in their half sites which explain the versatile and seemingly degenerate DNA sequence recognition by Pax proteins. Domain swap experiments between BSAP and Pax-1 demonstrated that the sequence specificity of the BSAP paired domain is determined by both its N- and C-terminal subdomains. Moreover, mutations affecting only one of the two subdomains restrict the sequence specificity of the paired domain (Czerny et al., 1993). Such mutations have previously been shown to be the cause of mouse developmental mutants (undulated, Splotch, and Small-eye) and human syndromes (Waardenburg's syndrome and aniridia) and may thus differentially affect the regulation of target genes by the mutated Pax protein.

The Pax-5 gene is expressed in tissues of three distinct origins during mouse ontogeny. In early development, Pax-5 is transiently expressed in the posterior midbrain and in the neural tube along the entire anterior-posterior axis. Later, it is expressed in all B-lymphoid tissues and in testes of the adult mouse (Adams et al., 1992). This expression pattern therefore suggests a role for Pax-5 (BSAP) in B-cell differentiation, neural development and spermatogenesis.

To examine the function of Pax-5 in these processes, we have inactivated the Pax-5 gene by homologous recombination in ES cells. For this purpose we have chosen to replace exon 2 with a lacZ gene and neo expression cassette (Fig. 2). Deletion of exon 2 is expected to inactivate Pax-5, as it eliminates the N-terminal part of the paired domain which is indispensable for DNA binding (Czerny et al., 1993). Three targeted ES cell clones were injected into mouse blastocysts and in all three cases high coat color chimERIC mice were obtained that transmitted the targeted Pax-5 allele to the germline. The analysis of Pax-5 (−/−) mice revealed that homozygous mutant animals are born at roughly the expected frequency. Hence, Pax-5 is not essential for embryonic development. Homozygous mutant mice become, however, severely growth-retarded soon after birth and they usually die in the third week. Only very few Pax-5-deficient males survive into adulthood and these mice proved to be fertile, thus indicating that Pax-5 is not essential for spermatogenesis.

**Fig. 2: Targeted disruption of the mouse Pax-5 gene.** Exon 2 coding for the N-terminal part of the Pax-5 paired domain was replaced with a lacZ gene and neomycin selection marker by homologous recombination in ES cell.

Pax-5 (−/−) mice exhibit a localized lesion in the midbrain. The most posterior part of the midbrain known as the inferior colliculus is virtually absent in the midline and is only present as a rudiment on both lateral sides in mutant mice (Fig. 3). Moreover, the folium of the cerebellum which normally borders on the inferior colliculus has expanded in the anterior direction to fill the space of the missing inferior colliculus. The inferior colliculus is considered to be an important relay site of the central auditory pathways that connect the cochlea of the ear with the primary auditory cortex of the brain. Contrary to expectation, Pax-5-deficient mice react, however, normally to noises and may thus suffer only from minor hearing defects, if at all.
B-cell differentiation is a highly ordered process in which progenitor cells undergo sequential differentiation steps characterized by rearrangement of immunoglobulin genes and by expression of stage-specific cell surface markers (Fig. 4). Histological analysis revealed that the spleen of Pax-5-deficient mice completely lacks lymphoid follicles which consist primarily of B-lymphocytes. In agreement with this finding, flow cytometric analysis indicated that all lymphoid tissues of Pax-5-deficient mice lack pro-B and mature B cells due to arrest of B cell development at an early progenitor stage. Pax-5-deficient mice contain only a low number of large CD43+ B220+ slgM+ cells which thus exhibit a phenotype characteristic of pro-B cells (Fig. 4). The block of B-lymphoid differentiation at the pro-B cell stage is complete, as no antibodies can be detected in the serum of adult Pax-5 (-/-) mice. Moreover, other hematopoietic lineages are not affected in mutant mice consistent with the B-lymphoid-restricted expression pattern of Pax-5. Taken together, these experiments revealed that Pax-5 plays an essential role in B-cell differentiation and normal midbrain development of the mouse.

Analysis of Pax-8 gene expression during mouse ontogeny

Zbynek Kozmik

Pax-8, a close relative of the Pax-5 gene (Adams et al., 1992), was previously shown to be expressed in the developing CNS, kidney and thyroid gland of the mouse (Pichlov et al., 1990). We have recently identified Pax-8 binding activity in several human kidney carcinoma cell lines. Four different Pax-8 mRNA isoforms, a-d, were subsequently cloned from one of these cell lines by PCR amplification and the Pax-8 gene was isolated from a human cosmids library. Analysis of the exon-intron structure of Pax-8 revealed that the four mRNA isoforms arise by alternative splicing, resulting in inclusion or exclusion of exon 7 and/or exon 8 sequences (Fig. 5). All four Pax-8 proteins retain the paired domain as their DNA-binding motif and recognize DNA in the same manner as the closely related Pax-2 and Pax-5 (BSAP) proteins. The Pax-8a and Pax-8b isoforms end in a serine/threonine/tyrosine-rich sequence, while the C-terminus of Pax-8c and Pax-8d is translated in a different, proline-rich reading frame. Transient transfection experiments revealed that Pax-8b isoforms and a, b, but not c and d, strongly stimulate transcription from a promoter containing six copies of a paired domain recognition sequence. The same four mRNA variants were also detected by RNase protection analysis in the mouse embryo and adult kidney, thus indicating evolutionary conservation of Pax-8 mRNA splicing. A different splice pattern was observed in the developing placenta, which expresses two new variants, Pax-8e and Pax-8f, instead of transcripts b-d. Expression of these mRNAs is high at embryonic day 9.5 and is gradually reduced until Pax-8a is the predominant transcript in the 12.5-day placenta. In the embryo, however, the synthesis of mRNAs b-d is initially low and then increases relative to that of Pax-8a. Hence, alternative splicing of Pax-8 gene transcripts not only generates six different Pax-8 variants, but is also temporally and spatially regulated during early mouse development (Kozmik et al., 1993). To elucidate the in vivo function of Pax-8, we have recently started to inactivate this gene by targeted disruption in the mouse genome.

human Pax-8

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

Introduction

The transcription factor AP-1, which is composed of dimeric complexes formed between Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) proteins, has been implicated in diverse cellular processes including cell proliferation, differentiation and neuronal function. In most cell types, the activity of this transcription factor is rapidly and transiently induced by a variety of extracellular signals. AP-1 is therefore thought to play a central role in signal transduction by reprogramming gene expression and thus coupling short-term signals elicited at the cell surface to long-term changes in cellular phenotype. AP-1 regulates transcription of target genes either by protein-protein interaction with other transcription factors or by binding to its DNA recognition sequence (known as AP-1 element; TGA GGT TCA) in promoter and enhancer regions (reviewed by Angel and Karin, 1991). To date, >20 different AP-1 target genes have been identified by defining functional AP-1 elements in their control regions by in vitro mutagenesis and transient trans-
fications. Most of these AP-1 target genes code for secreted proteins including growth factors, hormones and proteases [reviewed by Busslinger and Bergers, 1993]. To facilitate the identification of additional AP-1 target genes, we have previously developed two estrogen-dependent Fos induction systems which are based on the fact that o-Fos is a limiting component of AP-1 activity in cycling cells in contrast to the Jun proteins. Both induction systems allow selective activation of AP-1 by providing the limiting Fos component (Brauslmann et al., 1993; Superti-Furga et al., 1991) and thus facilitate the identification of transcripts which are regulated by AP-1 activity. In this manner, the coding for the transcription factor Fos-1, the membrane associated proteins annexin II and V and the biosynthetic enzymes tyrosine hydroxylase and omithin decarboxylase were shown to be Fos-responsive in fibroblasts and/or PC12 cells (Brauslmann et al., 1992; Wrighton and Busslinger, 1993). In addition, we have isolated a novel Fos-induced transcript (Fit-1) by differential cDNA cloning from rat fibroblasts expressing the estrogen-inducible Fos-ER fusion protein (Superti-Furga et al., 1991). During the last year we have pursued the characterization of the Fit-1 gene which provided new insight into how transcriptional regulation of a single gene can give rise to the synthesis of secreted and membrane-bound proteins.

The Fos-responsive gene Fit-1 codes for secreted and membrane-bound proteins related to the interleukin-1 receptor

Gabriele Berger, Arnold Reikerstorfer and Paula Graninger

The Fit-1 gene was shown to be directly regulated by the estrogen-inducible transcription factor Fos-ER and to belong to the family of delayed early genes which are dependent on protein synthesis for their induction by growth factors. Two different mRNA isoforms are expressed from the Fit-1 gene. The Fit-1M mRNA isolated from spleen codes for a membrane-bound protein which is most closely related in its extracellular, transmembrane and intracellular domains to the type I interleukin-1 (IL-1) receptor. The Fit-1S mRNA of fibroblasts directs, instead, the synthesis of a secreted protein consisting of only the extracellular domain. Analysis of the exon-intron structure of the Fit-1 gene indicated that the Fit-1S and Fit-1M mRNAs are transcribed from two different promoters and that the sequence differences at their 3' ends result from alternative 3' processing (summarized in Fig. 6). Northern blot analysis with specific 5' and 3' probes directly demonstrated tight coupling between alternative promoter usage and 3' processing of the Fit-1 transcripts. The homologous gene of the mouse (known as T1 or ST2) is expressed during ontogeny first in the fetal liver of the embryo and then in lung and hematopoietic tissues of the adult. The mRNA coding for the membrane-bound protein is more abundantly expressed in all of these tissues, while the transcript for the secreted form predominates in fibroblasts and mammary epithelial cells. Differential regulation of two distinct promoters is thus used to determine the ratio between secreted and membrane-bound forms of Fit-1 (T1/ST2) which may modulate signalling in response to IL-1. So far two different IL-1 receptor genes (type I and II) have been identified. We are currently performing IL-1 binding studies with Fit-1S and Fit-1M to see whether the Fit-1 gene codes for yet a third type of IL-1 receptor.

Fig. 6: Exon-intron structure of the Fit-1 gene and splicing of its transcripts. The common exons (C-9a) which code for the extracellular domain are indicated by black boxes. TM, transmembrane domain. For details see Berger et al. (1993).

Publications during the year


Other references


Cell Division and Differentiation in Yeast

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Introduction

Despite major advances in recent years, our understanding of how cells proliferate and differentiate, let alone how they choose between these two options, remains rudimentary. Four sorts of questions stand out: how do cells coordinate their growth and division, how do cells coordinate the duplication and segregation of their chromosomes, how do external signals influence cells to differentiate or divide, and how do cells sometimes give rise to progeny that behave differently? One of the major advances in cell biology of the past ten years has been the discovery that the architecture of eukaryotic cells and the regulation of their division is highly conserved. This means that fundamental questions can be addressed using organisms whose study is more tractable and the answers gleaned are valuable in directing subsequent human studies. Our current knowledge of cell cycle control has been pioneered by genetic analyses of two distantly related yeasts, the budding yeast Saccharomyces cerevisiae and the fission yeast Schizosaccharomyces pombe. Such studies have revealed the role of a highly conserved class of protein kinase dependent on regulatory cyclin subunits (the CDK kinases encoded by CDC28 and cdc2 genes). Cell cycle dependent fluctuations in different forms of these kinases, distinguished primarily by the type of cyclin subunit, play an important part in the alteration of S and M phases. Activation of the CDK kinase by G1-specific cyclins in late G1 triggers the initiation of DNA replication, whereas activation by G2-specific cyclins in G2 causes entry into mitosis (see Figures 1 and 2). Studies on the budding yeast have also shed important insights into the relationship between proliferation and differentiation. Haploid cells with opposite mating types cause each other to arrest in the G1 phase of the cell cycle and to differentiate into gametes that subsequently conjugate to form diploid zygotes. These pheromones activate a highly conserved cascade of protein kinases, the last of which (Fus3, a MAP kinase) phosphorylates and thereby activates an inhibitor (Far1) of G1 cyclins.

The cell cycle as a developmental process

The progression of CDK kinases during the cell cycle is analogous to alterations in gene activity that characterize embryonic, or indeed any other form of development. Different phases of development constitute the life cycle of an organism, just as G1, S, G2, and M phases constitute a cell division cycle (Fig. 1). Understanding each phase of an organism's development depends on knowledge about a preceding phase. For example, the establishment of stripes of gene activity during embryogenesis in Drosophila depends on gra-
Q1 - How do cells coordinate cell division with growth?

Most proliferating cells maintain a constant size at a given stage of the cell cycle, which is often cell type dependent; that is, their rate of cell division and mass doubling are coordinated. This is largely achieved by two fundamental properties of all eukaryotic cells: the ability to replicate and segregate chromosomes faster than doubling their contents (at least when cells become larger than normal) coupled with the dependence of S phase entry on a cell reaching a critical size (more likely a matter of achieving a certain rate of protein synthesis - see Fig. 3). What is the mechanism linking S phase entry with growth of the cell?

A critical cell size is needed for S phase entry

Fig. 3 Small daughter cells must spend longer than larger daughter cells growing large enough to initiate DNA replication.

Our current understanding of this process in budding yeast is that the transcriptional activation of the G1 cyclin genes, CLN1 and CLN2, only occurs when cells reach a certain size. However, we have identified the transcription factor (called SBF) responsible for activating these genes. We are currently studying how transcription due to SBF is activated in a size-dependent fashion by a third type of G1 cyclin gene, CLN3, whose transcription is not cell cycle regulated and whose protein therefore exists in small G1 cells. It is conceivable that cyclins D and E from vertebrates correspond to CLN3 and CLN1/2 respectively and that the activation of cyclin E by cyclin D is by analogy an important step in commitment to S phase in humans. We are also investigating the possibility that there exists in addition a size control over the activation of forms of the CDC28 kinase that are more directly involved in the initiation of DNA replication. The G1 cyclins encoded by CLN1 and CLN2 are involved in cell cycle commitment (known as Start, see Fig. 4), whereas other G1 cyclins, encoded by CLB5 and CLB6, are directly involved in the initiation of DNA replication (see Fig. 5).

Q2 - How do cells ensure that chromosome duplication precedes segregation?

It is currently thought that cells replicate their DNA before attempting to separate their chromosomes due to the activation of S phase promoting cyclin-dependent kinases (CDks) before M phase promoting CDks (Fig. 1). In vertebrate cells, the former differ from the latter in both cyclin and CDK kinase subunits, whereas in yeast, they seem to be distinguished solely by the type of cyclin subunit associated with a single CDK (cdc2/CDC28). How are oscillations in different cyclins (Fig. 2) determined? What turns on G1 cyclins in late G1 and turns them off in G2? What turns on G2 cyclins in G2 and causes their disappearance in mitosis? Furthermore, what ensures the correct order of appearance of different types; i.e. what prevents G2 cyclins appearing before G1 cyclins? This problem, which is known as the alteration problem, has long been considered to be one of the most fundamental problems in cell cycle control. The identification of cyclins and CDks is but the first step in its solution. We now must understand the dynamics of different CDks.

We are currently studying what restricts the accumulation of CLN1 and CLN2 CDK kinases to late G1 phases and of mitotic CLB1 and CLB2 CDK kinase to
Q2/M phases in the budding yeast *Saccharomyces cerevisiae*. The following crude picture is emerging: growth leads to an increase in CLN3 CDK kinase, which then activates transcription of CLN1 and CLN2. These enable CLB1 and CLB2 to activate their own synthesis (see below), and CLB1/2 CDK kinases then cause the repression of CLN1 and CLN2 and trigger the onset of M phase (see Fig. 6). Some event in mitosis then activates the proteolysis of CLB1 and CLB2 proteins, which leads to the inactivation of their synthesis and exit from M phase, which sets the stage for the re-activation of CLN1 and CLN2 by CLN3. We have recently discovered that CLNs are necessary for inactivating the proteolysis of CLBs started in mitosis, thereby ensuring that accumulation of CLBs starts prior to activation of CLNs (see Fig. 7). A major unknown in this regulatory cycle is what triggers the proteolysis of CLBs in mitosis. To gain insight into this phenomenon, we have isolated conditional lethal mutants which are defective in CLB2 proteolysis. Our screen employs a CLB2-LacZ fusion protein. Using this system, it should also be possible to isolate mutants in which proteolysis is deregulated, i.e., turned on at all stages of the cell cycle. Characterization of the genes identified by such genetic studies should provide clues as to the mitotic event responsible for triggering CLB2 proteolysis.

Q3 - What mechanism triggers the onset of S phase?

It is important to determine exactly which cyclins activate which key cell cycle events. Until recently, it was thought that activation of CLN1 and CLN2 in late G1 signals the onset of S phase. We have recently identified two new B-type cyclin genes, CLB5 and CLB6, whose deletion causes a highly specific delay in the onset of DNA replication. DNA replication normally occurs at the same time as bud formation and the duplication of spindle pole bodies. However, in clb5 clb6 double mutants, S phase occurs at least 30 min later than bud formation. Transcription of CLB5 and CLB6 is activated in late G1 and this has led us to propose that the appearance of CDC28 kinase activity associated with CLB5 and CLB6 cyclins may be the signal that triggers S phase (see Fig. 5). There are two outstanding problems with this hypothesis. First, S phase is only delayed in the absence of CLB5 and CLB6 and second, premature activation of CLB5 transcription does not advance S phase. We have shown that other B-type cyclin genes, such as CLB1, 2, 3, and 4, substitute for CLB5 and CLB6. S phase is delayed by several hours in the absence of six CLB genes. Using a tagged version of the CLB5 protein, we have shown that premature activation of CLB5, due to expression from the *GAL1*-10 promoter, causes the premature accumulation of CLB5/CDC28 kinase complexes, which have no kinase activity due to the presence of an inhibitory protein. Destruction of this inhibitory protein seems to be an important step in the onset of S phase.

Q4 - What distinguishes the activity of different types of cyclins?

Why are CLN CDK kinases good at promoting events that occur at Start, whereas CLB CDK kinases are good at promoting the formation and function of mitotic spindles? The answer presumably lies in their substrate specificity, about which we know very little for lack of important physiological substrates. Transcriptional regulation by the SBF transcription factor is one of the few processes in budding yeast whose regulation by CDK kinases is well characterized. The late G1-specific transcription conferred by SBF binding sites arises due to activation by CLN CDK kinases and repression by CLB CDK kinases. Thus different types of CDK kinase have opposing effects on the activity of SBF (see Fig. 6). Understanding this phenomenon promises to shed important insight not only into the specificity of CDKs but also on the role of phosphorylation in regulating transcription.

Q5 - How does the CDC28 kinase activate transcription in late G1 and then repress it later in G2?

Stage specific gene expression is an important aspect of cell cycle progression. A large number of genes are only transcribed transiently in late G1. These include cyclin genes such as CLN1 and CLN2, which are important for initiating the cell cycle, and CLB5 and CLB6, which are important for initiating DNA replication. Two different transcription factors are responsible for all late G1-specific gene transcription in yeast: SBF, which is composed of the SWI4 and SWI6 proteins, and MFB, which is composed of the MBP1 and SWI6 proteins.
Q6 - To what extent is a cell's cell cycle position determined by factors other than its spectrum of CDK kinases?

Fusion of G1 with G2 cells delays the onset of mitosis in the G2 nucleus. The G1 nucleus presumably causes the production in the common cytoplasm of a substance that can inhibit the activation of mitotic CDK kinases. Current thinking holds that early G1 cells express all known CDK kinases that are specific to this stage, suggesting that some other property of G1 cells is responsible for mitotic delay. Continued mitotic cyclin protein synthesis in G1 cells might contribute to this effect, but there might also be other factors involved. Recent work on yeast suggests that replication forks could play an important part in delaying the onset of mitosis in S phase cells. Replication complexes and not forks themselves could be the source of this inhibitory signal. Such complexes may well form long before the onset of S phase, possibly at the end of the previous mitosis. Their presence, say in G1 and S phase cells, or absence, say in G2 cells, could therefore be important in determining cell cycle state. Investigating this hypothesis should be possible using the wide range of yeast replication mutants now available. We have meanwhile embarked on a screen to isolate mutants which 'forget' that they have reached G2. Cells lacking the four mitotic cyclins CLB1-4 complete DNA replication but then fail to enter mitosis and arrest indefinitely in G2. We have recently isolated mutants which instead of arresting in G2 restart the cell cycle; these mutants both re-replicate their DNA and re-bud without any nuclear division. A further analysis of these mutants promises to identify new genes involved in cell cycle control.

Q7 - How does the cell cycle interface with differentiation?

Most instances of cellular differentiation are probably incompatible with simultaneous proliferation. Such incompatibility is most pronounced in cases of terminal differentiation where cells lose forever their ability to divide. Cell cycle events may be important for at least two aspects of differentiation. We presume that differentiation factors not only induce new patterns of gene expression but also arrest cell cycle progression. It is also likely that processes connected with cell division will be important for the asymmetric segregation of sister cells of factors that determine the subsequent cell fate of these cells.

Cell division in the yeast Saccharomyces cerevisiae occurs by budding and is inherently asymmetric. Furthermore the two progyn have different developmental fates: the mother cell expresses the HO endonuclease gene and as a consequence switches its mating type, whereas the daughter cell derived from the bud cannot express HO and retains its original mating type. By this means, a single cell can produce progeny with different mating types, which then arrest each other in G1 through the secretion of pheromones and subsequently conjugate. We are currently studying events in mitosis needed for activating HO in mother cells and have set up a screen to isolate mutants in which HO is expressed in daughter cells. Our study of HO's cell cycle control yielded insights into the biochemistry of Start, for example, identification of the SBF transcription factor now known to be important for the expression of CLN1 and CLN2. In a similar manner, we expect that the study of HO's mother cell specific expression will yield insights into aspects of the mitotic or cell division apparatus that are asymmetry.

Publications during the year


I. FUNCTION OF TRANSCRIPTION FACTOR AP-1

Over the past years, we have been involved in studies aimed at unraveling the function of AP-1 in vivo. The AP-1 transcription factor complex is comprised of a multi-gene family consisting of the products of the Fos-related (c-Fos, FosB, Fra-1, Fra-2) and Jun-related (c-Jun, JunB, JunD) genes. These proteins are thought to play an important role in cellular proliferation, differentiation, transcription and oncogenesis. The goal of these experiments is to define the biological roles of these proteins at the molecular level.

1. Analysis of Fos mutant mice

Agis Grigoriadis, Zhao-Qi Wang, Thomas von Rüden and Seiji Okada in collaboration with Marco Cecchini and Herbert Fleisch (University of Bern)

We have previously reported that mice lacking Fos are viable but develop a bone disease called osteopetrosis, which is characterized by impaired bone remodeling and a lack of tooth eruption. Fos mutant mice also exhibit extramedullary hematopoiesis and contain a reduced number of B lymphocytes (Wang et al., 1992).

Hematopoietic stem cells lacking Fos are pluripotent. Fos mutant mice were used to characterize in detail the developmental potential of hematopoietic stem cells lacking c-Fos and the mechanisms of the altered B cell development. In vitro colony assays and bone marrow reconstitution experiments demonstrated that Fos mutant hematopoietic stem cells can differentiate into all mature myeloid as well as lymphoid cells at a similar frequency to wild-type stem cells. Reconstitution of the bone marrow space of Fos mutant mice by transplantation of wild-type bone marrow cells also resulted in the concomitant rescue of the B cell defect. These results demonstrate that hematopoietic stem cells lacking Fos are apparently not affected and that the observed B cell lymphopenia is a consequence of the altered bone marrow environment due to the osteopetrosis.

Absence of osteoclasts in Fos-less mice. Bone remodeling is a tightly regulated process involving the interaction between bone-resorbing osteoclasts

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Fig. 1: Schematic overview of research projects

Growth control genes such as growth factors and their receptors together with molecules involved in signal transduction and in gene expression are essential components for the regulation of mammalian development (Fig. 1). We are interested in the function of such genes in normal mouse development, stem cell differentiation and oncogenesis. Although the function of genes is exerted at the cellular level, all our approaches are aimed at studying the role of specific gene products in the context of the intact mouse. For these studies we are performing "gain-of-function" (overexpression) and "loss-of-function" (gene inactivation) experiments and are using as powerful tools: (i) Transgenic mice produced by micro-injection of DNA into fertilized eggs; (ii) genetically-altered mouse embryonic stem (ES) cells introduced into mouse embryos and (iii) retroviral gene transfer into hematopoietic stem cells. Apart from the individual projects described below, we have also made some efforts in understanding the biological role of PAX-5 in development in collaboration with Pavel Urbanek and Meinrad Busslinger (see report by Meinrad Busslinger), and the role of p53 in hematopoietic cell development (in collaboration with Moshe Oren, Israel).
2. C-Jun is required for normal mouse development and hepatogenesis

Jun Liang, Ingrid Aschauer-Fedka and Norma Howells in collaboration with Frank Hildeg (Bender + Co.), Adriano Aguzzi (University of Zürich) and Karl Schellander (Vet. University, Vienna)

The proto-oncogene c-jun is the cellular homolog of \(\gamma\)-jun, the transforming oncogene of the avian sarcoma virus (ASV)-17. C-Jun encodes the major component of the AP-1 transcription factor complex and is expressed in many organs during mouse development and in the adult. We have previously shown that embryonic stem (ES) cells lacking c-Jun are viable and have a normal in vitro differentiation capacity, although c-Jun appears to be essential for tumor growth in vivo (Hilberg and Wagner, 1992). To better define the function of c-jun in development, targeted c-jun-/- ES cells were used to generate mice lacking c-Jun. Heterozygous c-Jun+/- mice appear normal, but embryos lacking c-Jun die at mid to late gestation, and often exhibit impaired hepatogenesis, altered fetal liver erythropoiesis and generalized edema. Interestingly, c-jun+/- ES cells can participate efficiently in the development of all tissues in chimera mice except to the liver, thereby suggesting an essential function of c-Jun in hepatopoiesis and development. In collaboration with Kurt Zafoukui (University of Graz) we are searching for the target genes responsible for the affected liver development.

To confirm that c-Jun is solely responsible for the observed embryonic lethality, we rescued the genetic defect with a c-jun transgene. Transgenic mice efficiently expressing deregulated c-Jun from an H2-K\(^{b}\) promoter developed during development as well as in adult mice exhibit no apparent phenotype. These were mated to c-jun+/- mice, and offspring were subsequently intercrossed to yield mice homozygous for the inactivated c-jun allele, but expressing the transgene. The rescued mice are viable and have reached adulthood exhibiting no major abnormalities, although they show erythroid abnormalities and reduced fertility with 100% penetrance. Currently we are characterizing these defects which may be caused by the inappropriate transgene expression in the rescued mice. These results suggest that c-Jun may have additional functions in other developmental processes besides hepatogenesis, which cannot be substituted by other AP-1 proteins. At present we are also attempting to rescue the deficiency in mice with a JunB transgene. In addition we are also defining the function of other Jun family members by generating mice lacking JunB (in collaboration with Marissa Schor and Peter Angl, Karlsruhe).

3. Functional analysis of the murine fra-1 gene

Martin Schreiber and Zhao-Qi Wang

Fra-1 (Fos-related antigen-1) is another important component of the AP-1 transcription factor complex. As a first step toward understanding the biological function of Fra-1 in vivo, we introduced a null mutation in the fra-1 gene by homologous recombination in ES cells. Several cosmids clones containing the fra-1 gene were isolated from a mouse genomic library and the sequence and exon-intron organization of the entire gene was determined. This information was used to construct a targeting vector in which the essential DNA-binding and dimerization (leucine zipper) domains of fra-1 are replaced by the bacterial genes.

4. Analysis of AP-1 function in vitro using fibroblasts lacking c-Fos and c-Jun

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

The generation of mutant mice provides an ideal source of cell populations for studying cell proliferation, transformation and gene function. Therefore, we have isolated primary embryonic fibroblasts from mice lacking either c-Fos, c-Jun or both proteins. Subsequently, spontaneously immortalized cell lines were established from these primary fibroblasts following the 3T3 protocol.

Whereas Fos-less fibroblasts exhibit only minor alterations in proliferation capacity, both primary and 3T3-like fibroblasts lacking c-Jun show markedly reduced growth rates. This is also reflected in the prolonged culture time required to immortalize these cells (Fig. 4), which is in marked contrast to Jun-less ES cells, where the growth rate is indistinguishable from that of wild-type ES cells (Hilberg & Wagner, 1992). Northern blot analysis of Fos-less fibroblasts and ES cells has shown that fra-1 expression is drastically reduced following serum stimulation when compared to wild-type cells. However, the expression levels of all other AP-1 family members analyzed were essentially unaltered. We are now extending these expression studies to Jun-less fibroblasts. With respect to functional studies, we have first analyzed the UV response, which is thought to be a c-Jun-dependent process. The results show that cell survival following UV irradiation is indeed greatly reduced in Jun-less cells compared to wild-type cells, and we are currently investigating whether other AP-1-dependent processes have also been affected.
IV. FUNCTION OF GROWTH FACTOR RECEPTORS

Growth factor receptors, in particular receptor tyrosine kinases, are known to play important roles in the regulation of cell proliferation and differentiation and their deregulated expression is often involved in the process of tumor formation. One of the best known members of the tyrosine kinase receptor family is the epidermal growth factor receptor (EGF-R), which binds multiple ligands including EGF and transforming growth factor-alpha (TGF-α). This receptor is expressed in mice as early as the 8-cell stage of development and in most tissues of the adult animal. This broad prenatal expression pattern suggests that the EGF-R might have important functions in mouse development and cell differentiation. However, very little is known about the mechanisms by which these receptors influence the growth and differentiation of specific cell lineages. To investigate the function of growth factor receptors, we expressed ectopically the normal human EGF-R (HER2) and mutant derivatives in primary mouse bone marrow cells and in ES cells. In tumors, we are analyzing the function of the EGF-R and the insulin-like growth factor II receptor (in collaboration with Denise Barlow) by inactivating these genes in ES cells and in mice.

1. Ectopic expression of normal and mutant epidermal growth factor receptors during mouse hematopoietic development

Thomas von Rüden and Laura Stingl

Retroviral gene transfer of normal and mutant EGF-Rs was established enabling their expression in murine long term reconstituting hematopoietic stem cells. In vitro experiments showed that these receptors can function on primary mouse bone marrow cells stimulating proliferation of mast cells. Expression of mutant EGF-Rs (v-erbB and a chimeraic EGF-Fv-erbB receptor) in bone marrow recombinant mice leads to the development of a severe mast cell disease, reminiscent of malignant mastocytosis in man, and in a few cases, to myelogenous leukemias (von Rüden et al., 1992). These results indicate that normal and mutant EGF-Rs transduce mitogenic signals and may even transform hematopoietic cells. Finally, ectopic expression of these receptors rescued the mast cell defects but not the stem cell defect in W/W mutant mice which are devoid of the c-kit-expressed Steel factor receptor (SLF-R). It appears that normal and mutant EGF-Rs substitute for certain functions of the SLF-R suggesting that there may exist partially overlapping pathways for different receptor tyrosine kinases.

2. Repression of exogenous growth factor receptor expression

Ulrike Burkert and Marion Fung

To analyze how growth factor receptors may influence the process of stem cell proliferation and differentiation, we attempted to express receptor tyrosine kinases in ES cells. For these studies we have used the normal human EGF-R (HER2), its constitutively active retroviral counterpart v-erbB, and the recombinant chimeric receptor mutants HERvRb, HERvR-flm and HER-c-kit. In the receptor chimeras the intracellular tyrosine kinase domains of the v-erbB and v-fls onco-genes and the human c-kit proto-oncogene are regulated by the ligand binding region of HER2. Various heterologous vector constructs, using the inducible human metallothio- nione promoter (MT), the constitutive PGK-promoter, as well as retroviral vectors (LEN and NTK), were used for gene transfer into ES cells. High frequencies of receptor-positive clones were only obtained with the v-erbB oncogene (Fig. 6A). Cell surface expression of HERvRb and HERvR-flm occurred at very low frequency (1/30 to 1/40 G418-resistant clones) and no ES cell clones were identified that expressed the HER-c-kit or the HER2 receptors.

To study the effect of exogenous receptors on stem cell differentiation several mutant ES clones were induced to differentiate in vitro or in vivo by induction of terato- megaly. Various heterologous vector constructs, using the inducible human metallothioneine promoter (MT), the constitutive PGK-promoter, as well as retroviral vectors (LEN and NTK), were used for gene transfer into ES cells. High frequencies of receptor-positive clones were only obtained with the v-erbB oncogene (Fig. 6A). Cell surface expression of HERvRb and HERvR-flm occurred at very low frequency (1/30 to 1/40 G418-resistant clones) and no ES cell clones were identified that expressed the HER-c-kit or the HER2 receptors.

Recently, we have shown that EGF-R expression is not due to rearrange- ment at the site of DNA integration, nor does it correlate with the methylation profile of the vectors. In summary, exogenous expression of receptor tyrosine kinases in ES cells is inefficient and occurs only at low frequen- cies; it is unstable and is lost upon differentiation both in vitro and in vivo. The mechanism underlying this repression is not understood at present.

3. Generation of cells and mice lacking EGF-R

Marie Sibilla

To elucidate the function of EGF-R during mouse development the gene was inactivated by homologous recombination in ES cells. Several λ clones that contained EGF-R genomic sequences were isolated and parts of them were used to construct a targeting vector in which the lacZ gene expression is driven by the endogenous EGF-R promoter. This construct was introduced in various ES cell lines and correct target- ing was achieved at a frequency of 1 in 5 G418-resistant clones (Fig. 6B). Various clones of each ES cell line were injected into C57BL/6 blastocysts and several highly chimeric mice were born. We are currently testing these chimeras for germline transmission to obtain EGF-R deficient mice which will be used to study both the expression pattern and the consequen- ces of the loss of EGF-R during development. We are also trying to inactivate the second EGF-R allele in ES cells using a hygromycin targeting vector. The availa- bility of these cells should provide us with a powerful tool for probing the role of EGF-R in multiple cellular processes such as signal transduction, cell differen- tiation and tumor development.
4. Inactivation of the insulin-like growth factor II receptor gene
Marion Fung and Zhao-Qi Wang in collaboration with Denise Barlow

The insulin-like growth factor II receptor, also known as the large mammalian 6-phosphate receptor (IGF2/MPR) is expressed both during mouse development and in the adult animal. This receptor plays a role in regulating IGF-II levels in body fluids and in intracellular lysosomal targeting. However, its full biological function is complex, since this receptor also binds other growth factors such as latent TGF-β and prolinlein. The IGF2/MPR gene has been shown to be imprinted in mice and is possibly responsible for the embryonic lethality associated with the Tme mutation (see report by Denise Barlow).

To investigate its biological role, we have inactivated the IGF2/MPR gene by homologous recombination in ES cells. Consecutive inactivation of the second allele was achieved at a very low frequency by selection at high concentrations of G418. We have generated chimeric mice which have transmitted the inactivated allele to offspring and are now in the process of analyzing in detail the phenotype(s) e.g. embryonic lethality of IGF2/MPR deficient mice. In addition, we will investigate the expression pattern and will derive cell lines from mutant mice to analyze the role of IGF2/MPR in lysosomal targeting, and to establish whether the lack of this receptor is causally related to lysosomal storage disorders (see also report by Denise Barlow).

V. GENERATION OF "ES MICE"
Zhao-Qi Wang and Friedemann Kieler

Chimeric mice generated by blastocyst injection of ES cells contain a mixture of derivatives from both ES cells and host embryos. However, completely ES-derived fetuses can be generated following aggregation of ES cells with tetraploid host embryos. Although these mice die at birth for unknown reasons, a new ES cell line (R1) has been isolated recently which gives rise to live newborns, albeit at a low efficiency (Nagy et al., 1993; F. Kieler, 1993). Tetraploid embryos can be obtained by electrofusion of 2-cell embryos, which are developmentally restricted and only form extraembryonic tissues at later stages of development. Since it is difficult to obtain ES cell-derived mice by aggregation using genetically-modified ES cells, we developed an alternative approach to generate "ES mice". Preliminary results show that completely ES-derived mice can be obtained which survive to adulthood. Therefore, this method should be useful for analyzing gene function in hematopoietic development, for gene expression analysis during embryogenesis as well as for shortening the time to generate mice with predetermined genetic changes. Currently we are testing this technique for its usefulness to study gene function in vivo following gain-of-function and loss-of-function mutations.

VI. ROLE OF p53 IN NORMAL AND LEUKEMIC BLOOD CELL DEVELOPMENT
Thomas von Rüden in collaboration with Eyal Gottlieb and Moshe Oren
(Weizmann Institute, Israel)

Hematopoiesis is a highly dynamic process in which cells constantly proliferate, differentiate and/or die. This process is mainly regulated by growth factors and cellular interactions. Programmed cell death (apoptosis) is a major mechanism of eliminating cells in several developmental processes. Apoptosis can be inhibited by mutant forms of the tumor suppressor gene p53, e.g. in myeloid leukemic cells and in factor-dependent hematopoietic cell lines. To assess the role of wild-type p53 in mediating apoptosis following the withdrawal of survival factors, we interfered with the endogenous p53 activity in an IL-3-dependent hematopoietic cell line (DA-1). Extended survival in the absence of IL-3 was conferred by recombinant retroviruses encoding either a full-length p53 mutant (ts p53 val135) or a C-terminal mini-gene, both of which act as dominant-negative inhibitors of wild-type p53. In contrast, excess wild-type p53 failed to induce apoptosis as long as IL-3 was present. We propose that p53 is a positive, although not exclusive mediator of survival factor dependence in hematopoietic cells.

References


Further references


Regulation of Protein Serine / Threonine Kinases During Early G1

Introduction

Addition of growth factors to non-growing (G0) cells leads to a rapid increase in protein phosphorylation and entry into the G1 phase of the cell cycle. Some of the proteins that become phosphorylated presumably have important regulatory functions and their activities change upon phosphorylation. A key problem in the area of signal transduction has been to identify these phosphoproteins and the kinases that phosphorylate them, and to determine how the kinases are regulated.

Several mitogen-activated kinases have been identified, including MAP kinases and S6 kinases. S6 kinases are serine/threonine-specific enzymes that phosphorylate the S6 protein of 4OS ribosomal subunits. These kinases are inactive in G0 cells but immediately become activated when cells are exposed to mitogens and enter G1. During this time S6 becomes highly phosphorylated and there is an increase in protein synthesis that is required for cells to progress into S phase. Two distinct families of S6 kinases have been identified: the 70 kDa enzymes (M_S=65-86 kDa), which are the major S6 kinases in mouse fibroblasts, and the 90 kDa S6 kinases, also called rsk (M_S=85-92 kDa). Enzymes in the two families share several properties: they are activated in vivo by growth factors, phorbol esters, oncogene products, etc.; activation requires phosphorylation of serine/threonine residues; and phosphorylation inhibits the enzymes in vitro.

On the other hand, p7056k and p9056k display different protein structures, distinct kinetics of activation, and, most important, they lie on different signalling pathways (Sturgill et al., 1988; Ballou et al., 1991). In particular, p9056k is activated by MAP kinase, but p7056k is not (Fig. 1). We are interested in identifying the cellular mechanisms that regulate the activity of p7056k and want to determine if the enzyme plays a role in controlling the cell cycle.

Identification of the S6 kinase activator

Lisa M. Ballou and Christian Kühne

In analogy to the pathway leading to activation of p9056k (Fig. 1), it appears that p7056k participates in a phosphorylation cascade, with at least one serine/threonine-specific kinase lying between growth factor receptor kinases and S6 kinase. We are using several strategies to identify the putative S6 kinase kinase, with the ultimate aim of finding all the components in this signalling pathway. The yeast 2-hybrid system is being used as a genetic approach to identify proteins that physically interact with p7056k in vivo. We have isolated positive clones from a human lymphocyte cDNA library and are now analyzing them. These cDNAs may encode enzymes that regulate S6 kinase (i.e., S6 kinase kinase, S6 kinase phosphatase, etc.) or they may yield new substrates for the enzyme. In conjunction with the genetic approach, we are also exploring biochemical means to identify the S6 kinase kinase. This continues to be a tough project: it is known that there are at least 4 mitogen-responsive phosphorylation sites in the p7056k molecule (Ferrari et al., 1992), so full activation of the enzyme could be a complex process requiring phosphorylation by several different kinases acting in a specific order. Instead of using minute amounts of dephosphorylated p7056k purified from fibroblasts in an in vitro reactivation assay, we have now expressed large amounts of wild-type and mutant forms of p7056k in insect cells using the baculovirus system. These proteins have been purified and are being used as substrates to look for enzymes that phosphorylate them. Finally, we found that the apparent size of p7056k increases when it is activated.

This could be due to an association with its activator. We are testing this hypothesis by looking for proteins that co-immunoprecipitate with the kinase from in vitro-labeled cells.

Inhibitors of S6 kinase activation

Claudia Petritsch (in collaboration with Peter Parker, ICRF, London)

Another approach to identify components in the p7056k pathway is to study the mechanism of action of inhibitors of the pathway. Thapsigargin is best known as a non-specific phosphodiesterase inhibitor that elevates cAMP and cGMP levels in cells. Pretreatment of fibroblasts with thapsigargin and related compounds completely blocked the phosphorylation and activation of p7056k but had no effect on p9056k or MAP kinase. Thapsigargin also caused a decrease in S6 kinase activity when it was added after the mitogen. These data suggested that these compounds either activate a phosphatase that negatively regulates the pathway or inhibit a kinase that positively regulates the pathway. Our initial hypothesis to explain the effect of thapsigargin was that high cyclic nucleotide levels activated a cyclic nucleotide-dependent kinase, which then phosphorylated an enzyme in the p7056k cascade to inhibit it. However, experiments using cyclic nucleotide analogs, specific phosphodiesterase inhibitors, and a cell line deficient in cAMP-dependent protein kinase indicated that cyclic nucleotides and cyclic nucleotide-dependent kinases probably do not play a role in the inhibition of S6 kinase by thapsigargin. We are now exploring alternative mechanisms that could explain the mode of action of thapsigargin: (a) it could directly bind to and inhibit a specific kinase in the p7056k pathway; (b) it could act by deregulating Ca2+ levels through the opening of ryanodine receptor Ca2+ channels; or (c) it could act by antagonizing adenosine receptors.

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Fig. 1: Signal transduction pathways leading to the activation of p9056k (reviewed in Pelech, 1993 and McCormick, 1993) and p7056k. Protein kinases are shown in bold face. Light arrows indicate hypothetical interactions and heavy arrows indicate direct interactions.
Rapamycin is an immunosuppressant that also specifically blocks the p70S6k pathway. Like theophylline, rapamycin inhibits phosphorylation of p70S6k, but it is not known whether the two inhibitors affect the same phosphorylation sites on the enzyme. Genetic experiments in yeast suggest that rapamycin inhibits a lipid kinase that is structurally related to mammalian phosphatidylinositol-3 (P13) kinase (Kunz et al., 1993).

Cell cycle regulation of S6 kinase
Helga Edelmann

Although it is known that p70S6k is activated as much as 50-fold during the G1-S transition, it is not yet clear whether this activity is essential for cell cycle progression. On one hand, microinjection of antibodies that inhibit p70S6k into fibroblasts at any time during G1 blocked entry into S phase (Lane et al., 1993); on the other hand, rapamycin prevented T cells from entering the cell cycle but did not arrest cells that were already in G1, even though the kinase was inhibited (Terada et al., 1993). This suggests that the function of the kinase may be different during G1-S, as compared with G1 in cycling cells, and this difference may be cell-type specific. Another question is whether p70S6k is active at other times during the mitotic cell cycle. To address these questions, we examined the behavior of the kinase in synchronized fibroblasts. We found that it was activated 20-fold in G1 cells treated with mitogens; the enzyme activity then decreased slowly until it reached a minimum at M phase, then was reactivated to a smaller extent when cells reentered G1. This regulation was mediated primarily through the phosphorylation state of the enzyme, as protein levels did not change significantly. We also examined the activity of MAP kinase and found that it displayed a pattern that was distinct from that of p70S6k. We next plan to measure cell cycle and cell growth parameters in cells in which S6 kinase is inhibited (by rapamycin, theophylline, dominant negative kinase mutants, etc.) or abnormally activated (by a constitutively active kinase mutant).

Publication during the year

Other references

Mammalian Developmental Genetics

mouse mutants as tools to isolate developmental control genes

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Introduction

Normal development of mammalian embryos is absolutely dependent upon implantation into the uterus and formation of a placenta that directly transfers maternal assets to the embryo. Mammalian embryos are thus very different from other animal embryos that develop independently to a feeding stage using yolk stored in the egg. Implantation, manipulation of maternal resources and intra-uterine growth are thus features unique to the mammalian embryo and therefore likely to be encoded either by novel genes or by genes that have acquired novel functions or expression patterns. Consequently, predictions about genes regulating maternal-fetal interactions cannot be made by studying other animal embryos and a molecular analysis of these events requires a direct analysis of the mammalian embryo.

The mouse is currently the best model for studying mammalian embryogenesis and mouse mutants whose phenotype demonstrates a clear developmental abnormality can provide the key that allows entry into regulatory molecular networks, once the mutant gene has been cloned. We have used "positional cloning" techniques to target two developmental mutations that fortuitously lie close together on chromosome 17; the first is the Tmc1 mutant which is the only known imprinted mutation in the mouse (imprinting may be one of the specific adaptations of mammals to allow for intra-uterine development and is explained in more detail below), the second mutation is called p70s6k and is essential for implantation.

The presence of genes in the mammalian genome that control embryonic implantation and embryonic manipulation of maternal resources very likely increases the disease load of the adult. For example, the controlled invasion of maternal tissue by the fetus, the transfer of maternal resources to the fetus and the regulation of fetal growth are all processes that involve genes that have the potential to cause severe disturbance to adult biological systems when inappropriately expressed. Thus the study of genes that control normal implantation and intra-uterine growth has great potential to identify genes involved in abnormal pathology. It is a concurrent goal of our research to analyze the potential of mammalian embryonic control genes to contribute to adult disease.
THE FUNCTION OF GENOMIC IMPRINTING IN MAMMALIAN EMBRYOGENESIS

Mammals inherit one set of chromosomes from each parent and are therefore, with the exception of genes on the sex chromosomes, genetically diploid. Recent discoveries, however, have highlighted the fact that although mammals are diploid, a mechanism known as genomic imprinting can regulate gene expression in a parental-specific manner generating phenotypic haploidy at a small number of autosomal gene loci. The molecular basis of genomic imprinting, while largely unknown, suggests that homologous gene loci are "marked" in a manner that allows them to be distinguished by the regulatory machinery within the diploid cell nucleus. Genomic imprinting has mostly been shown to affect gene expression and to act in both directions so that genes have been identified that are exclusively maternally expressed and some identified that show exclusive paternal expression. Very recent findings which show that replication timing and chromosome translocations can also be regulated in a parental-specific manner, suggest that the effects of imprinting could extend beyond regulation of gene expression. Genomic imprinting, which is defined as a parental-specific difference in chromosome or locus behavior, is not restricted to mammals and occurs in plants, yeast and insects. However, only in mammals do imprinted genes play a role in the developing embryo. The action of imprinted genes in this context is still largely unknown, and it is clear that their role in development is complex and not yet fully understood.

Our efforts over the last few years have succeeded in identifying an imprinted gene (Barlow et al., 1991) that is closely linked to the only known imprinted mouse mutation known as Tme (T-associated maternal effect). This gene has been identified as the Insulin-like growth factor type 2 receptor, a bi-functional receptor also known as the cation-independent mannose 6-phosphate receptor, now abbreviated to lgt2/MPR. We are currently testing in collaboration with the laboratory of Erwin Wagner at the lgf2/MPR gene in the Tme mutation by analyzing mice lacking this gene through homologous inactivation. More recent work identified a region within the mouse lgt2/MPR locus that carries an imprinting mark (Stüger et al., 1993) and proposed a model (see below) describing the origins of genomic imprinting in mammals (Barlow, 1993). Current projects, described below, are concerned with testing our proposed model of imprinted gene regulation in mice and humans and with utilizing the imprinted region to isolate other imprinted genes from the mouse genome.

Fig. 2: A model for genomic imprinting
1a. Is the Tme mutant a mouse model for human lysosomal disease?
D. P. Barlow, K. Nolan*, in collaboration with members of Erwin Wagner's laboratory
(* University College, Dublin)

The Igf2/MPR gene was initially identified through its role as a lysosomal targeting receptor, a component of the intra-cellular system that transports proteins to lysosomes. Defects in various components of this pathway have been identified in humans and a large number of lysosomal storage disorders have been characterized (Nolan & Sly, 1989). These disorders are not readily treatable and often fatal in early adolescence. The Tme (T-associated maternal effect) mutant is a classical mouse mutation that carries a 3 cM deletion of chromosome 17 that removes an essential embryonic gene, which we have suggested is the Igf2/MPR gene. Igf2/MPR is expressed exclusively from the maternally inherited chromosome (Barlow et al., 1991) and embryos with a deletion of the expressed maternal gene copy show an increased body size generally attributed to severe edema. Death occurs between E15.5 and E 17.5 (Johnson, 1974) In an attempt to correlate this phenotype with the role of the Igf2/MPR gene we are conducting an analysis of lysosome enzymes and lysosomal function in cell lines established from Tme mutant E13.5 embryo fibroblasts. Results from the Tme fibroblasts will form a basis for a subsequent analysis of tissues from the Igf2/MPR "knock-out" mouse (see also report from E. F. Wagner's laboratory) and establish if mice lacking the Igf2/MPR receptor can be used as a model for human lysosomal storage disease.

1b. The molecular basis of the imprinting signal
Pavel Kubicka, Anton Wutz and Heinz Himmelbauer

Parental-specific gene expression that is a result of genomic imprinting is thought to be generated by a modification placed on only one chromosome that negatively or positively interferes with gene expression. These and other characteristics of imprinted gene expression suggest that the imprinting modification is epigenetic and reversible on passage through the germ line, have led to the proposal that imprinted gene expression is regulated by DNA methylation. We have recently searched the mouse Igf2/MPR locus for a methylation imprinting mark and identified an element, named "region 2", that fits these criteria (Stöger et al., 1993). Two of the most interesting features about region 2 are that it is located in a downstream intron (27 kb from the transcription start) and that it is methylated only on the expressed locus (Fig. 3).

Fig. 3: A methylation imprint at the mouse Igf2/MPR locus

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These features suggest that region 2 acts to modify transcription and that methylation is, contrary to expectation, necessary for expression of this gene. Thus region 2 could either be an enhancer or a repressor binding element. A positive correlation between methylation and expression could arise via the action of a methyl-dependent enhancer protein (that binds to the expressed locus) or through the action of a methyl-sensitive repressor protein (that binds to the silent, non-methylated locus). These predictions are being tested by an analysis of factors that bind in vivo and in vitro to region 2 in the methylated or unmethylated state.

Transgenic mice are being used to assess the role of region 2 in imprinting the Igf2/MPR locus. Reporter constructs derived from either cosmids or YACs (yeast artificial chromosomes) that contain different genomic elements (Fig. 4) are introduced into the mouse germ line by microinjection. Cosmid MVIII/B is derived from a 39 kb cosmId and contains the Igf2/MPR promoter fused to a 5-globin termination signal. Cosmid BC is 54 kb long and contains these two elements plus region 2. The YAC reporter constructs have the advantage of containing the complete Igf2/MPR gene plus flanking genes. YAC fragmentation and homologous recombination have been used to genetically engineer these constructs in yeast. The effect of parental inheritance upon methylation and expression are monitored by breeding the transgenic animals.

COSMIDS
MVIII/B

BC

YAC-TS

Fig. 4: Cosmid and YAC transgenic reporter constructs

1c. Comparison of genomic imprinting between different species
Oskar Smrcka

Several genes have been shown to be imprinted in both mice and humans but it is still open whether the corresponding human IGF2/MPR gene shows features similar to those seen in mice. Therefore, the finding of a human homolog to region 2, which we propose to be the imprinting box, could help to define an inter-species consensus sequence and the essential characteristics of this functional region. Should the imprinting mechanism of the mouse Igf2/MPR gene be conserved, the corresponding imprinting box of other species is expected to be, like the mouse region 2, CpG rich and differentially methylated.

It has also been shown that imprinting of embryonic control genes can play a role in pathogenesis. One example is the human IGF2 gene of which monoallelic expression is lost in Wilms' tumor, a phenomenon that has been named "Loss of Imprinting". Other examples in which imprinting plays a role in human disease include growth defects, abnormal neuronal development and chromosomal rearrangements in leukemogenesis. For this reason human homologs of other newly identified imprinted mouse genes will be characterized in order to study their role in physiological and pathological mechanisms.
1d. A genome wide search for imprinted genes

Björn Neumann

Previous analysis of the mouse Igd/Mpr-gene revealed that a sequence within the second intron (termed region 2) may carry the imprinting signal that causes exclusive expression of the maternally derived gene. Methylation of region 2 serves as a biochemical marker to differentiate between parental genomes. A sequence analysis of this methylated region led to the identification of a CG rich stretch of DNA, that appears identical with CpG islands. It has a CG/CpG ratio of 1 and is 1500 bp in size. Within the first 800 bp of this island a number of iterated direct repeats can be detected by computer analysis which are unrelated to the major LINE and SINE repeat families (Fig. 5). A computer search of other sequences known to be imprinted again revealed sequences with similar characteristics to the region 2 type imprinting box, i.e. CG richness, iterated repeat structures and allele-specific methylation.

The general strategy for a genome wide search for imprinted genes will therefore rely on the common features of methylation and base content. This can be done by using a pair of restriction enzymes with one isoschizomer being methylation sensitive while the other does not distinguish between the methylated and unmethylated restriction sites. In addition, the recognition sequence of the endonucleases should be CG rich. The enzyme pair HpaII and MspI meets these criteria. The goal for this approach will then be to clone small pieces of DNA (as theoretically small fragments are preferentially released from CG rich genomic regions following the above scheme). After excluding repeat sequences, clones are analyzed for differential methylation in the genome and the presence of direct repeats before being tested for exclusive parental specific expression of the gene using an RNA RFLP.

Investigated region showing differential methylation

1 - 4 indicate the identified structural organization of the repeats

Fig. 5: Structural features of region 2

GENES THAT REGULATE THE INVASIVE PROPERTIES OF THE MAMMALIAN EMBRYO TROPHOBLAST

Norbert Schweller, in collaboration with the laboratory of Hans Lehrach, ICRF, London.

The aim of this project is to use an implantation defective mouse mutant to isolate genes responsible for the invasive properties of the mammalian embryo trophoblast. Identification of the mammalian embryo is an invasive process that requires a balanced interaction between two tissues - the embryonic trophoblast and the maternal uterus. The genes that regulate the invasive behavior of embryonic tissue have not been identified, but it is predicted that similar genes may also play a role in the behavior of invasive tumors. Thus an understanding of the molecular basis of embryonic implantation may elucidate events in tumor pathology.

Homoeologous mouse embryos bearing a mutation at the recessive m7 locus cannot implant and subsequently die 8-9 days after fertilization. The trophoblast of this mutant apparently acts normally during attachment to the uterine wall, but is considered to be deficient in invasive ability from histological studies (Axelrod, 1985). Fortuitously the m7 mutant has been mapped as lying within 300 kb of the Tme locus on chromosome 17, thus our analysis of Time has provided tools with which to access the m7 gene (Schweller & Barlow, 1992). Our attempts towards the goal of cloning m7 have led to the identification of a novel sequence which is currently under analysis.

Transmission of genetic information from parent to offspring requires the segregation of chromosomes from diploid stem cells into haploid gametes. This reduction of chromosome number is achieved by the meiotic cell division and ensures that each gamete carries one copy of each chromosome type. Furthermore, these haploid cells must undergo a differentiation process which renders the gamete competent to participate in fertilization. The process of meiosis and meiotogenesis is performed by germ cells in the specialized environment of the testes in the male and ovaries in females. Insight into the process of chromosome segregation was first provided by Mendel's first law of segregation. Mendel stated that each homologue of a chromosome type stands an equal chance of being transmitted to the next generation. However, in 1922 a variant form of mouse chromosome 17 was identified which distorts the Mendelian ratio of transmission. This variant (known as the I haplotype) is inherited from the male parent with greater than 95% of offspring at the cost of its meiotic partner, the wild type form of chromosome 17. This phenomenon is referred to as transmission ratio distortion and is an example of meiotic drive. While a number of meiotic drive systems are known, especially in invertebrates, the mouse system is unique in that the loci responsible for the distortion are mutant forms of genes essential for male fertility. As such, they provide a rare opportunity to investigate meiosis and male-specific gametogenesis in a mammalian organism.

The genetic analysis of the I haplotype revealed that 4 interacting loci control the meiotic drive phenomenon. These loci reduce the fertility of gametes inheriting the wild type form of chromosome 17 while allowing the I haplotype-bearing gametes to retain fertility. Because these loci are implicated in meiotic-specific sterility, it has been suggested that meiotic drive may involve hybrid sterility genes. This would imply a role for these loci in the process of speciation. We have used positional cloning to identify one of the meiotic drive genes located at the Toc-3 locus (I complex distiller 3). This gene named Tcto-2 (I complex testes expressed) is only expressed during male meiosis. Genomic and cDNA cloning has shown that the locus is in excess of 100 kb and expresses multiple, differentially spliced mRNAs of varying sizes. Only one of these mRNAs contains an open reading frame and its role in meiosis and meiotic sterility is currently being investigated.
Group Barlow

Publications during the year


Other references


Group Cotten

Virus Entry Mechanisms and Gene Delivery

Group leader Matt Cotten
Chief technician Medjha Saltik
Postdoc Susanna Chiocca
PhD student Adam Baker

1. General Overview

An underlying theme in our group is an effort to understand adenovirus entry into host cells as well as to mimic this process in strategies to deliver DNA to the nucleus of mammalian cells. A number of barriers to successful DNA delivery are described in fig. 1. Improvements in DNA delivery systems will only come with an improved understanding of the basic chemistry of DNA/protein interactions, of endocytosis, phagocytosis and the dynamics of the vesicular system of cells - because these are the entry routes into the cells - and of transcriptional control mechanisms, so that we can ensure gene expression once our DNA is within the target cell. In addition, an understanding of DNA repair and integration mechanisms is warranted because these phenomena most certainly will be involved in integrating the new DNA into host chromosomes and in maintaining the gene in dividing cell populations. One goal of these studies is to produce improved DNA delivery methods that can be applied in clinical settings (see Cotten and Wagner, 1993 for a review).

2. Recombinant, endosomolytic particles that are useful for gene delivery

In the most efficient forms of receptor-mediated gene delivery, developed in collaboration with the groups of Max Birnstiel and Ernst Wagner, polylamine-condensed DNA is coupled to adenovirus particles with the virus providing a useful endosome disruption activity (Wagner et al., 1992; Cotten et al., 1993; see reports from the Max Birnstiel and Ernst Wagner groups for additional details). We are working to replace the
adenovirus with simple, nonviral endосомото-момо-момо-е
ments. The most efficient endosome disrupting agent, adenovirus, presents membrane active peptides in a highly ordered structure and perhaps the presentation of endosomes peptides on a protein with the ability to self-assemble into ordered particles might provide a better reagent for augmenting gene delivery. As the starting scaffold for these particles, we identified viruses with simple, single protein capsids that can self-assemble into ordered structures. We then modified the genes encoding these proteins to include membrane-disruptive peptides. Expression of the proteins, purification and assembly into particles should yield the desired particles (Fig. 2). Two structures have been investigated for this purpose: the endogenous retroviral element of yeast, Ty and the bacteriophage MS2. In both examples, inserting the pH-dependent membrane active GALA peptide (Subbarao et al., 1987; Parente et al., 1990; Wagner et al., 1992b) into a surface-exposed site in the capsid protein yielded recombinant particles that are active for DNA delivery (Chiocca and Cotten, 1994). A number of additional membrane-active peptides as well as ligand peptides are now being investigated.

3. Empty adenovirus particles and the proteolytic activation of endosomalytic activity.

The adenovirus particle is a potent reagent for enhancing receptor-mediated gene delivery, yet we do not fully understand, at the molecular level, how the virus passes through or disrupts membranes as it enters cells. Part of the research of our group has been an effort to understand the adenovirus entry mechanism, including the role of proteolytic processing in activating the endosomalytic activity of adenovirus.

During adenovirus virion assembly, a shell of the major capsid proteins is first assembled and then filled with the viral DNA. If the virus capsid is responsible for the membrane disruption during virus entry, it appeared reasonable to purify these relatively DNA-free empty particles and use them for gene delivery experiments. We have found, however, that these empty capsids are defective for gene delivery and this defect is due to the absence of the pH-dependent membrane disruption activity of the virus capsid. During virus assembly, entry of the viral DNA provides a signal to activate a viral encoded protease. This protease cleaves a number of capsid proteins and this cleavage is required for the generation of mature, infectious virus particles (Weber, 1976). In collaboration with Joseph Weber, (University of Sherbrooke) we have demonstrated that particles from a temperature sensitive protease strain of adenovirus are defective for gene delivery and for membrane disruption when grown at the restrictive temperature (Cotten and Weber, 1994). We are studying this phenomenon in more detail to learn about the viral proteins involved in the membrane disruption and cellular entry process.

4. Inactivation of adenovirus particles to eliminate virus replication and transcription while maintaining the endosomalytic activity of the virus capsid.

In collaboration with Malgorzata Kuras and Ernst Wagner (Bender) as well as Gerd Maass and Max Birnstiel, we have analyzed methods of damaging the adenovirus DNA genome without disrupting the entry mechanisms of the virus capsid. The entry enhancement is a function of the viral capsid; viral gene expression or viral DNA replication is not required for the gene delivery enhancement. Therefore, the utility of the adenovirus-augmented gene delivery system would increase if we could ensure the absence of viral gene expression or replication. As an alternative to empty capsids, we have pursued methods of inactivating the viral genome of mature particles. We have previously described a psoralen/UV treatment of viral particles that produces a greater than 5 log decline in the cytopathic effect of the virus without disrupting the DNA delivery and cellular entry functions of these virus particles (Cotten et al., 1992). Psoralens can enter a number of different types of viral particles and upon 365 nm UV irradiation, form covalent inter- and intra-strand adducts with the viral DNA or RNA (Hanson, 1992, see fig. 3). These adducts block virus transcription and replication. We have compared adenovirus inactivation with several different psoralen derivatives as well as the more traditional virus inactivating agent β-propiolactone. No replication-competent adenovirus can be detected in β-methoxypsoralen-inactivated preparations. However, following β-propiolactone treatment, a small amount of virus genome remains intact and replication competent virus can be detected at a low frequency. Northern analysis of gene expression from the inactivated virus demonstrates that both β-methoxypsoralen and β-propiolactone block virus gene expression (E1a and E3) to the same extent. Analysis of virus gene expression by RT-PCR demonstrates that RNA synthesis from psoralen-inactivated virus is inhibited at least 1000-fold relative to non-inactivated virus. Consistent with the inhibition of viral RNA synthesis and the block to virus replication, labeling adenovirus with 3H-labeled, β-methoxypsoralen shows that the psoralen adducts are distributed throughout the virus genome at a frequency of 1 adduct per 800 basepairs of DNA.

Fig. 2: Self-assembling recombinant endosomalytic particles

Fig. 3: Protein inactivation of adenovirus
5. LPS toxicity in the presence of adenovirus

Endotoxin (lipopolysaccharide, LPS) is commonly found as a contaminant in plasmid DNA preparations either purified by CsCl or by ion-exchange techniques. The LPS molecule is an extremely potent stimulator of the mammalian immune system, in collaboration with Ernst Wagner (IMP and Bender) and Michael Buschle (Bender) we have found that the quantities of LPS typically contaminating DNA preparations are toxic to primary cells in the presence of entry-competent adenovirus particles (see fig. 4, Cotten et al., 1994). Simple and effective methods of removing the contaminating LPS using either a polynyx resin or Triton X-114 extraction have been identified. Treatment of DNA samples to remove LPS eliminates the toxicity to primary cells. It is not yet clear if the toxicity is due to physical damage to the cell by the LPS, or if the combined interaction of adenovirus particles and LPS molecules activates a toxic signalling pathway. Experiments to clarify the mechanism generating this toxicity are in progress.

![LPS toxicity in the presence of adenovirus](image)

Fig. 4: LPS is toxic to primary human melanoma cells in the presence of adenovirus particles. Primary human melanoma cells were exposed to the indicated quantities of LPS and adenovirus d1014, psoralen-inactivated. After 48 hours, the cell samples were washed once with HBSS, fixed in formaldehyde and the surviving cells visualized by crystal violet staining.

6. The measurement of transfection complex size by light scattering.

Dynamic light scattering (DLS) can provide a rapid (5 minute) determination of particle sizes in the 30-1000 nm size range. We are using the DLS technology to measure adenovirus/DNA complexes and artificial virus complexes. The ease of particle size determination should facilitate efforts to streamline our DNA delivery complexes.

Publications during the year


Other references

Chiocca, S. and Cotten, M. (1994). Recombinant endosomolytic particles based on the MS2 capsid can be used for gene delivery to mammalian cells. (manuscript in preparation).


Molecular Mechanisms of Locus Control and Chromatin-Dependent Gene Expression

Introduction

Differential gene activity is the basic principle to establish cell type determination, which in turn is required to generate a multicellular organism. The goal of my research is to gain insight into the molecular mechanisms that govern the establishment of these cell-type-specific patterns of gene expression. In contrast to our advanced understanding of transcriptional stimulation, my research plans focus on the less well defined mechanisms on how chromatin-dependent alterations in gene expression patterns promote or perturb mammalian cell type specialization and development. Using the immunoglobulin heavy chain (\(\mu\)) enhancer and murine lymphoid differentiation as a model in transgenic mice, my specific aims are: (i) to dissect molecular mechanisms of locus control and (ii) to isolate mammalian regulators of the chromatin structure.

I. Molecular mechanisms of locus control

Expression of immunoglobulin genes is tightly regulated and starts around day 15 of mouse development in the fetal liver. Expression in the adult animal is restricted to lymphoid organs (spleen, lymph nodes and thymus). This tight stage- and tissue-specific expression pattern, combined with an extensive analysis of the respective regulatory sequences, has made immunoglobulin gene expression an excellent model system to study developmental control of gene activity.

(a) The accessibility problem

The immunoglobulin heavy chain (\(\mu\)) enhancer region confers high transcriptional activity upon the \(\mu\) gene in multiple chromosomal locations in transgenic mice. The general dependence of transgene expression on enhancers or on enhancer-like locus control regions (LCRs) suggests a role for these sequence elements in initiating factor access in nuclear chromatin. Studies addressing this putative role, however, are complicated by the functional interaction of enhancers and LCRs with promoters for RNA polymerase II. In particular, it remained unclear as to whether enhancers and LCRs can function at the initial step of "programming" a specific region in chromatin for factor access prior to the onset of transcription. To directly demonstrate the function of enhancers in establishing accessibility, I have analyzed factor access in nuclear chromatin independent of the presence of a linked RNA polymerase II promoter and independent of ongoing transcription.

The experimental strategy involved the microinjection into fertilized mouse eggs of minigenes consisting of the binding site for the prokaryotic T7 RNA polymerase (T7RNAP) and various fragments from the \(\mu\) enhancer region. In contrast to stable transfections in tissue culture, this mode of gene transfer has the following two advantages: (i) it allows the test constructs to be assembled into chromatin during normal development and (ii) it does not require the presence of an expressed dominant selectable marker gene that could obscure alterations in the chromatin structure. The accessibility of the introduced T7 promoter can subsequently be examined by the addition of purified T7RNAP to nuclei from immortalized transgenic pre-B cells that were derived from fetal liver by infection with Abelson murine leukemia virus (A-MuLV). Since the sequence-specific binding of monomeric T7RNAP to its promoter does not depend on the interaction with other proteins, T7 promoter recognition by T7RNAP is a direct function of the accessibility of this particular nucleotide sequence and can be quantitated relatively easily by measuring the synthesis of T7-specific run-on transcripts (see Fig. 1).

(b) Architecture of a locus control region

Using this "T7 accessibility assay", I was able to demonstrate that a specific combination of factor binding sites within the \(\mu\) enhancer core is sufficient to "program" the chromatin structure for localized factor access independent of measurable transcription (Jenuwein et al., 1993a). Subsequently, a multi-step model for the generation of an active transcriptional state of a gene locus has been proposed in which A/T-rich matrix-attachment regions (MARs) are likely to be required to propagate this localized factor access to distal promoters and to generate a "hot" sensitive chromatin (Jenuwein et al., 1993b). According to this model, the combination of transcription factor binding sites with flanking MARs may be important to constitute a functional LCR to confer long-range alterations in chromatin (see Fig. 2).

To test the generality of this model, I intend to examine a variety of locus control elements (e.g. present in the \(\beta\)-globin or lysozyme loci) for similar functions. Using gene cassettes containing T3 and T7 promoters linked to wild-type and mutated LCR fragments at proximal and distal positions, the following questions will be addressed: (i) what are the minimal sequence requirements for MAR function? (ii) is a specific alignment combination of MARs and transcription factor binding sites required? (iii) how far from a LCR can alterations in chromatin be propagated?

(c) Chromatin composition and factor access

Alterations in chromatin structure have frequently been correlated with changes in transcriptional activity. Transcriptionally active chromatin has been shown to be highly acetylated at histone H4, enriched in HMG proteins and to exhibit a great reduction in the amount of H1. However, the cause-and-effect relationship between these alterations in chromatin composition and transcription remained unclear because of the difficulty in uncoupling the events that initiate changes in chromatin from those of transcriptional activity itself. Likewise, the question arises as to whether the regulation of factor access depends upon alterations in chromatin composition. To address these questions, cross-linked chromatin derived from transgenic pre-B cell lines carrying transcriptionally inactive \(\mu\)T7 transgenes that differ in their potential to establish accessibility will be enriched by immunoprecipitation with antibodies specific for HMG/Y or acetylated histone H4. Subsequently, DNA isolated from this cross-linked "active chromatin" fraction will be visualized by DNA blot analysis specific for \(\mu\)T7 transgene sequences. These experiments will directly address (i) whether extended accessibility (see Fig. 2) is preferentially correlated with "active chromatin" and (ii) whether an altered chromatin composition can be induced independently of the process of transcription.
LOCUS CONTROL IS A MULTI-STEP PROCESS

II. Mammalian regulators of the chromatin structure

The packaging of DNA into chromosomal domains and the decondensation of higher order chromatin are efficient mechanisms to regulate the activity of key developmental loci in eukaryotes. Examples include the mating type loci in yeast and the homoeotic loci in Drosophila. The "on-or-off-state" of these developmentally regulated loci is usually reset during the very early stages of development as a consequence of active restructuring of chromatin. The genetically stable remodeling of the chromatin structure induced by enhancers and locus control regions is very likely to depend upon similar mechanisms to regulate stage- and tissue-specific gene expression.

(a) Stage- and tissue-specific regulators of the chromatin structure

Our knowledge of mammalian gene products interacting with chromatin to regulate locus control elements is limited. By contrast, the powerful genetics available in yeast and Drosophila facilitated the isolation of genes whose products appear to be directly involved in mediating chromatin-dependent changes in gene expression. Both activators (e.g. SNF2 and bmr) and repressors (e.g. SIR1 or aux1-201) of enhancer function in chromatin have been described. I intend to isolate murine counterparts with degenerate oligonucleotides specific for domains conserved between yeast and Drosophila chromatin regulators. In particular, I plan to screen cDNA libraries specific for early lymphoid development (for putative activators), fibroblastic cells (for putative repressors) and embryonic stem cells (for general, non tissue-specific regulators). Candidate cDNAs can then be analyzed for their function in expressing regulation of defined lymphoid-specific marker genes in transfection studies in tissue culture and, more directly, with gene targeting experiments.

(b) Chromatin regulators at a specific LCR

Although interesting tissue-specific mammalian regulators interacting with chromatin can be identified using the protocol outlined above, it may prove difficult to demonstrate their involvement with specific locus control elements. Therefore, in a more direct approach, I also plan to isolate regulators of chromatin structure through complementation cloning in yeast. This seems particularly suited, since proteins restructuring chromatin do not seem to bind to a specific DNA sequence but rather interact with regulatory proteins already sitting at the target site. A paradigm for chromatin-dependent gene expression is represented by the yeast mating type locus, MAT. It has been suggested that changes in the chromatin structure allow expression at the accessible MAT locus but repress activation at the flanking silent loci HMLa and HMLb. I intend to introduce a lacZ reporter gene under regulatory control from parts of the µ enhancer into yeast strains by targeting it into the accessible MAT locus, or into one of the inaccessible silent loci. Activation of the lacZ reporter through the subsequent expression of mammalian transcription factors specifically interacting with binding sites in the µ enhancer should therefore be conditional upon the respective chromatin structure. Although the chromatin structure in yeast is less complex than that of a higher eukaryote, these yeast strains will be extremely useful to screen the above mentioned cDNA libraries for candidate gene products. A direct demonstration of their role in regulating a specific locus control element (e.g. µ enhancer) through alterations in chromatin can then be done with gene targeting experiments in mice.

(c) Remodeling the chromatin structure in vivo

The stable inactivation of clusters of genes through heterochromatinization or the activation of genes by decondensing higher order chromatin represent important control points to reprogram patterns of gene expression during early development. However, little is known about the mechanism(s) that underly heterochromatinization. Recently, murine cDNA clones were isolated whose products are enriched in euchromatin (e.g. HMG1/2) or heterochromatin (e.g. HP1). I plan to offset the balance between euchromatin and heterochromatin by introducing null mutations of HMG1/2 and HP1 in embryonic stem cells and mice. These studies will advance our understanding of (i) the mechanism(s) that form euchromatic and heterochromatic domains during early mouse development and (ii) allow the direct examination of the functional roles of these "general" coregulators of the chromatin structure in controlling gene activity.

Publications during the year


Suggested reading


Periodic Transcription during the Yeast Cell Cycle

Introduction

The investigation of the cell cycle has led to a general concept of how a cell regulates its progression through the cell cycle (Fig. 1). Currently, it is thought that a serine/threonine kinase, p34^cdk2, 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 homolog 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 p34^cdk2 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. At a result of this event, the cyclin subunits are thought to be released from the complex and destroyed. Experiments in S. pombe, marine invertebrates and now also in S. cerevisiae have shown that in G2, before mitosis, p34^cdk2 complexes with G2 cyclins to form an active kinase of probably a different specificity, whose activity then leads, after a phosphorylation and dephosphorylation step, 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. The initial question for our research at the IMP was 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. Such genes exist is supported by recent findings which show that the G2 cyclin genes as well as most of the G1 cyclin genes in S. cerevisiae are cell cycle specifically regulated. The constitutive overexpression of at least some of these genes leads to cell cycle defects in a growing yeast cell. Also, transcriptional activation of G2 cyclin genes in the G2-stage of the cell cycle, that leads to the formation of mitotic kinase, is required to repress G1 cyclin gene expression. These results suggest that cell cycle dependent expression of important cell cycle controlling genes is a necessity for the cell's progression through its life cycle. The isolation of novel cell cycle regulated genes could lead to the identification of yet unknown genes which are involved in important cell cycle processes and which may also be homologous to genes in higher eucaryotes exhibiting homologous function.

Regulation of MST2

Axel Behrens and Tillman Schuster

The two genes MST1 and MST2 encode highly related type 3 membrane proteins. Since they belong to the class of proteins that possess seven membrane domains they could represent G-protein coupled receptors (Fig. 2). The similarity of these proteins with bacteriorhodopsin, however, suggests that they could also function as ion channels.

Both genes are cell cycle dependently regulated. They were the first genes isolated from S. cerevisiae whose expression peaks in mitosis. Therefore, we became interested in studying how their expression depends on the events that control this cell cycle stage. This should result in the finding of new regulatory components that are involved in controlling the expression during mitosis.

We have shown that MST1, in contrast to MST2, is not only cell cycle regulated, but also super-induced by heat shock. To avoid possible problems which may be caused by interference with the heat shock response we chose MST2 to examine its promoter for possible regulatory elements. We hooked the MST2 promoter in front of the bacterial lacZ gene to use it as reporter gene. Nested deletions in the promoter region allowed us to define a 125 base pair region which is involved in activating the gene. Currently, we are in the process of defining this region more precisely.

We have also shown that the repression of MST2 transcription in the G1-stage of the cell cycle is dependent on G1 kinase function. In a yeast strain lacking the three G1-cyclins (cln1-, cln2-, cln3-), which arrests in G1, MST2 is still expressed. Only after releasing the cell from its arrest by using a GAL promoter driven CLN3 gene MST2 is switched off immediately. But in its G1 arrest MST2 expression can also be repressed by addition of the pheromone u-factor. This suggests that the promoter contains an element that is able to bind a transcriptional repressor which is activated by u-factor. We are currently searching for such an element.
Generation of synthetic lethal mutants of a \textit{kin3}-yeast strain
Wilfried Roesler and Tillman Schuster

\textit{kin3} encodes a putative Ser/Thr protein kinase which is closely related to \textit{nim3} (= never in mitosis), a gene of the filamentous fungus \textit{Aspergillus nidulans} that is required for progression into mitosis (Osmani et al., 1991a, b, c). We have shown that \textit{kin3} is regulated in a cell cycle dependent manner and that expression of \textit{kin3} peaks in mitosis. The \textit{kin3} gene is not essential for growth but its overexpression is lethal and leads to severe defects in cytokinesis and nuclear division. The cells do not separate any more and grow into long "worm like" structures.

The fact that the deletion of \textit{kin3} has no effect on growth may be due to the existence of a homologous gene which complements a \textit{kin3} functional defect. This makes it very difficult to analyze \textit{kin3} for the functional role it may have in division control. Therefore, we employed a colony color assay to screen for synthetic lethal mutations in yeast cells with no functional \textit{kin3} gene in the genetic background. We identified several mutants which depend on ectopic expression of \textit{kin3}. This enables us now to study \textit{kin3} function under genetic conditions where it has become an essential gene. In an initial experiment we transformed one synthetic lethal mutant with a genomic yeast library. We identified five different clones which could complement the mutant phenotype. One plasmid carried the \textit{kin3} gene. The other clones were found to overlap and contain the gene \textit{CCR4} (= carbon catabolite repressor; Malvar et al., 1992) which is required for the expression of genes involved in nonfermentative growth. It is thought, however, that \textit{CCR4} plays a very general role in transcription and is not only involved in carbon catabolite repression. Currently, we are examining the phenotype of the synthetic lethal mutants in the absence of \textit{kin3} expression.

An old transcription factor with a new job: \textit{Swi5} activates \textit{EGT2} in early G1 stage of the cell cycle
Bransilav Kovacevich and Tillman Schuster

In a comprehensive screen for cell cycle regulated genes involved in cell division control in the yeast \textit{Saccharomyces cerevisiae} (Price et al., 1991) we identified a gene which appeared to be transcribed in early G1 and which we named \textit{EGT2} (Early G1 Transcript). Since our work is directed toward the identification of cell cycle regulated genes with a possible role in the regulation of the cell cycle, \textit{EGT2} became a candidate gene to study. The aim of this work is to characterize the \textit{EGT2} gene, i.e., to identify the regulator(s) of its transcription and to determine the function of the gene product. \textit{EGT2} encodes an unknown protein with a length of 1003 amino acids. It contains a potential 20 amino acid leader peptide at its N-terminus and eight 35 amino acid repeats of a novel type at its C-terminal half (Fig. 3). Its deletion leads, after normal completion of cytokinesis, to a delay of cell separation in G1. This was shown by FACS analysis together with microscopic investigation of \textit{egt2} cell cultures.

\textbf{EGT2}

\begin{center}
\begin{tabular}{c}
\textbf{amino acids} \\
\textbf{TSSTDEGSYVSITSTESAQPYEVGSLLVPSTIKTS}
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\textbf{Consensus}

The presence of a leader peptide and some hints we got from in situ immunofluorescence studies prompted us to check whether the gene product is secreted. Comparing Western blots of proteins secreted from cells carrying either a functional or a non-functional version of \textit{EGT2} shows that only the functional protein is successfully processed and the N-terminal part is secreted. Next, we want to characterize the processing pathway of the \textit{EGT2} gene product and elucidate its function in cell separation.

The regulation of \textit{EGT2} transcription has been studied by hybridizing temporal staged RNA blots with radioactively labelled \textit{EGT2} probes. To avoid misinterpretation of the results three different methods were used to synchronize cell populations. In the first two cases we used the release from pheromone arrest in G1 and the release of a cdc15 mutation arrested in mitosis by incubation at the non-permissive temperature. In the third case we used cells harbouring the genotype cdc17, cdc2, ch3 which is rescued by a functional \textit{CNL1} gene driven by a GAL1 promoter. They were arrested in G1 by culturing in raffinose containing medium and then synchronously released by adding galactose. The results we got from these experiments lead to the conclusion that \textit{EGT2} transcription begins very late in mitosis (or immediately after it) and is repressed before the cell reaches START, the pheromone arrest point in G1.

The case that \textit{EGT2} is not expressed in the first cell cycle after release from the pheromone arrest appeared to be reminiscent of the transcriptional regulation of the HO gene that codes for a site specific endonuclease involved in mating type switching. Therefore, we tested whether \textit{Swi5}, one of the transcriptional activators of \textit{HO}, is also the activator of \textit{EGT2}. Our results show that in \textit{swi5} cells the level of \textit{EGT2} transcription is significantly decreased. Furthermore, in cells lacking both \textit{Swi5} and its homolog \textit{Ace2} (Butler et al., 1991), \textit{EGT2} transcription was completely repressed. The gene product of \textit{ACE2} activates the chitinase encoding gene \textit{CTS1} in a cell cycle dependent manner (Dohrmann et al., 1992). To explain how \textit{Swi5} functions so that it is able to activate two different genes, \textit{EGT2} and \textit{HO}, at different cell cycle stages, we developed a model. This model suggests that \textit{EGT2} is activated as soon as \textit{Swi5} enters the nucleus in early G1 while \textit{HO} is induced in late G1 only when additional transcription factors (Swi4/Swi6) contribute to \textit{Swi5}-activity. Our data of experiments using \textit{swi5} mutant strains tested at different cell cycle points prove this model.

\textbf{Publications during the year:}

\textbf{Other references}


Introduction

The IMP groups of Max Birnstiel, Matt Cotten and our group have developed a gene transfer system which uses the receptor-mediated endocytosis route to import DNA into mammalian cells (Cotten et al., 1993a and references therein). DNA gene constructs have been complexed with (i) a polylsine-conjugated ligand (such as transferrin, EGF, or monoclonal antibodies) for uptake into intracellular vesicles (endosomes) via the receptor-mediated endocytosis; and (ii) polylsine-conjugated, endosome-disruption agents (such as replication-defective and psoralen/UV- inactivated adenoviruses) which allow cytoplasmic entry of the delivered DNA. These complexes have been delivered to and expressed at high level in a large proportion of cultured target cells (up to 80% in primary fibroblasts, primary myoblasts or primary human melanoma cell cultures). The high gene-transfer efficiency in cell culture is contrasted by a poor efficiency in vivo; the DNA complexes are inactivated by serum components such as complement factors (our unpublished results). Nevertheless, the gene transfer technology is attractive for gene therapies where cells can be modified ex vivo. In collaboration with Bender we are developing a cytokine gene-modified melanoma vaccine (see below).

1. Synthetic virus-like gene transfer systems containing endosome-disruptive peptides derived from influenza virus sequences

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

The release of endocytosed DNA from internal vesicles has been shown to be a major limiting step in receptor-mediated gene delivery. We have demonstrated that viral entry into cells and subsequent escape from intracellular degradation can be mimicked by fully synthetic DNA complexes (Wagner et al., 1992; Plank et al., 1992). A variety of synthetic peptides (most of them homologous to the N-terminal fusion sequence of influenza virus hemagglutinin subunit 2, HA-2; see Fig. 1) were examined for their membrane-disruptive capacities in liposome leakage and erythrocyte lysis assays.

The peptides disrupted phosphatidylcholine-liposomes in a pH- and concentration-dependent manner (high activity at acid pH, low or no activity at neutral pH). Interestingly, peptides homologous to the N-terminal twenty amino acids of HA-2 (elongated with a short spacer sequence) turned out to be inactive in the erythrocyte lysis assay, whereas dimers and polymers of these peptides displayed high pH-specific activities. The question arose whether a cooperative action of two domains of influenza peptide head-to-head dimers (INF3D1 to INF3D2) would be sufficient for inducing lysis of natural membranes or whether an increased length of the amphipathic sequence would be necessary. Chimeric peptides GALA-INF1 and GALA-INF3 composed of the first 16 residues of the influenza sequence (and derivatives thereof) elongated with 18 amino acids of the artificial membrane-disruptive GALA sequence (Parente et al., 1990) displayed erythrocyte-lysing activities almost as high as the head-to-head influenza dimers. We also found that the elongation of the HA-2 sequence 1-20 with the subsequently highly conserved amino acids Trp-21, Tyr-22 and Gly-23 was sufficient to lead to the highest erythrocyte-lysing activity of all examined peptides (INF6, INF7; Fig. 1). In order to demonstrate that the influenza peptides also disrupt endosomes, cultured cells were incubated with 70 kD FITC-dextran in the presence of peptide. We could show the peptide-dependent release of counter-differentiated FITC-dextran from internal vesicles into the cytoplasm by fluorescence-microscopy (Plank et al., 1994).

For us the major incentive to examine such peptides is their use as endosome-disrupting agents in receptor-mediated gene delivery. We constructed a virus-like gene transfer vehicle by incorporating the membrane-disruptive peptides into DNA complexes by ionic interaction as shown in Fig. 2. Highly efficient delivery of DNA to a variety of cell lines could be achieved using such DNA complexes (Fig. 3). These results make us confident that in the near future synthetic virus-like but virus-free gene transfer vehicles will represent an efficient alternative to virus-containing gene transfer systems in gene therapy.
2. Different behavior of human rhinoviruses HRV2 and HRV14 in virus-augmented gene delivery

Wolfgang Zauner and Ernst Wagner, in collaboration with Dieter Blasch and Ernst Küchler (Vienna Biocenter, Institute of Biochemistry)

Several classes of viruses enter their host cells by receptor-mediated endocytosis accompanied by a destabilization of the endosomal membrane and a release of the viral genome or of whole particles into the cytoplasm. This mechanism, employing endosome-disruptive adenovirus particles, has been used for receptor-mediated gene delivery systems (Colten et al., 1993a). A drawback of using adenovirus or other DNA viruses for endosome disruption consists in the large amount of viral DNA that is co-delivered into the cell. Thus we used human rhinoviruses (HRVs), picornaviruses with a small RNA genome of about 7500 nucleotides, for the augmentation of receptor-mediated gene transfer. Two different serotypes, one belonging to the major receptor group (HRV14) and one to the minor receptor group (HRV2), were able to enhance gene transfer to murine NIH 3T3 cells, which are non-permissive for viral replication but express the HRV minor group receptor. For efficient gene transfer both viruses had to be incorporated into the transferin/DNA complex via biotin/streptavidin linkage (Fig. 4 A and B). Incorporation of up to 800 ng HRV2 or 1500 ng HRV14 increased reporter gene expression more than 1000-fold.

We next tested the effect of several known inhibitors of infection, such as chloroquine, monensin, bafloycin A1, and of the antiviral compounds WIN 1 (Ia) and WIN IV on the transfection levels in NIH 3T3 cells (Fig. 5 A and B). For both serotypes the addition of the lysosomotropic compounds chloroquine or monensin had only minor effects on reporter gene expression indicating that the DNA/virus complexes are able to penetrate into the cytoplasm in the presence of these drugs. The presence of bafloycin A1, a specific inhibitor of V-ATPase, resulted in a complete loss of reporter gene expression, suggesting that DNA release was completely inhibited. The two serotypes, however, behaved differently in the presence of WIN 1 (a) and WIN IV, two inhibitors of uncoating. While HRV2-mediated transfection was only marginally impaired, HRV14-mediated gene transfer was nearly abolished. For both serotypes WIN VIII, an inactive control compound, had no effect on reporter gene expression. Thus, several compounds known to inhibit viral replication have only a minor effect on gene transfer. Therefore we have been able to successfully transfect HeLa cells, which are permissive for viral replication.

![Diagram](image_url)

**Fig. 2:** Complexes of DNA, transferin-polylysine conjugates and endosome-disruptive peptides either covalently (Peptide-pLys) or immuno-bound to polylysine (pLys, Peptide).

**Luciferase Activity (Light Units)**

<table>
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<th>6</th>
<th>10</th>
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*Fig. 3:* Gene transfer to BNL C1.2 cells using complexes containing pCMV, DNA, transferin-polylysine, polylysine, and optimized amounts of indicated peptides.

**Fig. 4:** HRV2 and HRV14 enhanced gene transfer to NIH 3T3 cells. The luciferase activities, which were obtained with 50000 cells in the presence of the indicated amounts of bodytiblated HRV2 (A) or bodytiblated HRV14 (B) are shown.
The major function of the viruses in receptor-mediated gene transfer is thought to be a destabilization of the endosomal membrane. There is evidence that the endosome disruptive activity of picornaviruses resides in the N-terminal part of VP1, one of the capsid proteins (Frick & Hogle, 1990). Therefore we synthesized a peptide derived from the N-terminus of VP1 of HRV2. This peptide was able to disrupt artificial and natural membranes in a pH-dependent manner as shown by liposome and erythrocyte leakage assays. The peptide, which carries a negative net charge, could be incorporated into DNA/TtP complexes via ionic interaction with an excess of polylisine. The resulting complex increased transfection to NIH 3T3 cells to a level seen with biotinylated HRV2 (Fig. 6A). Chloroquine had only a minor influence on transfection levels, while monensin reduced the level to about 40%. Addition of bafilomycin gave nearly background levels of reporter gene expression. Uptake of the complex via the transferrin receptor was shown by competition experiments with an 120-fold excess of transferrin as well as with complexes assembled without transferrin (Fig. 6B).

Our data demonstrate that human rhinoviruses are able to efficiently enhance gene transfer by a destabilizing effect on the endosomal membrane. Efficient gene transfer to NIH 3T3 as well as HeLa cells is possible under conditions which are known to inhibit viral infection. With this system we are able to differentiate between HRV-mediated endosome disruption and infection; we hope that we can thereby elucidate the steps for viral infection in more detail.

3. Gene transfer into T-lymphocytes via anti CD3 antibody - DNA complexes

Michael Buschle, Matt Cotten, Helen Kirakos, Karl Mechtler, Max L. Birnstiel and Ernst Wagner

We have been studying the role of different cell-binding ligands for receptor-mediated gene transfer. In most previous experiments the transferrin has been used, but the Tf receptor density does not always correlate with the gene transfer efficiency. In T-cells, transferrin has been found to be a modest ligand, although T-cell lines express high levels of TfR. T-cells also express the 19-29 kDa surface protein complex CD3 which is closely associated with the T-cell receptor (Weiss, 1993). CD3 is present on greater than 95% of circulating human peripheral T-cells, expressed at a high density of 10,000-40,000 sites per cell. Binding of anti CD3 mAbs to the CD3/TCR complex is mitogenic for T-cells (Verwilghen et al., 1991) and results in rapid endocytosis of the CD3/TCR complex (Krangel, 1987).

Plasmid DNA encoding for a luciferase reporter gene, complexed with either polylisine, transferrin-polylysine, or polylisine-conjugated anti CD3 monoclonal antibodies was introduced into several T-cell lines as well as isolated primary blood lymphocytes. The expression levels obtained by using polylisine conjugates of anti CD3 are up to more than 1000-fold higher than those obtained with transferrin, unmodified polylisine, or other putative ligands for T-cells, such as anti CD7 or anti CD4. The transfection efficiencies correlate with the ratio of intracellular uptake of the complexes (see Fig. 7). Both anti CD3- and anti CD7-complexes bind to the cell surface, but only anti-CD3 DNA complexes are efficiently internalized. DNA complexes containing anti CD3 mAbs and biotinylated, replication-defective adenovirus particles show lower expression levels also as in primary lymphocytes. The system is currently applied to the expression of biologically more relevant proteins, such as exogenous cytokines, where an expression level of 90,000 pg interleukin-2/24 hours/million H9 cells was obtained.

Fig. 7: Binding of anti CD3- or anti CD7- DNA complexes to Jurkat cells results in efficient internalization of anti CD3 DNA complexes only (left: fluorescence microscopy; DNA stain with DAPI; right: phase contrast; arrows indicate DNA of complexes).

4. Receptor-mediated gene transfer in cancer therapy

Michael Buschle, Christian Pfirst, Wolfgang Zauner, Karl Mechtler, and Ernst Wagner, in collaboration with the group of Max L. Birnstiel, and Matt Cotten (I.M.P), Georg Stinigl and Susanne Schreiber (2nd Clinic of Dermatology, Vienna), and the Tumor Vaccine group at Bender, Vienna

The delivery of genes to tumor cells is considered to be an exceptionally well-suited application of transferrin receptor-mediated gene delivery. Rapidly proliferating tumor cells can be transfected with highest efficiency, which may partially reflect the high levels of transferrin receptor in these cells. But also slowly-dividing or non-dividing cells are accessible for receptor-mediated gene transfer. One might consider using the system to deliver toxins directly into the tumor mass for elimination of these cells (Cook et al., 1994; Curiel et al., 1994). However, the poor survival of our complexes in the blood stream coupled with the requirement to introduce the gene into most cells of the tumor limits this application.

Recent advances in the understanding of tumor immunology have promoted the idea of a tumor vaccine (Zelloukal et al., 1993). The concept is as follows: after surgical removal of the tumor from patients with e.g. high risk primary melanoma, a primary culture is transfected with one or several cytokine genes. X-ray irradiation blocks cell division and the tumor cells, which continue to produce cytokines, are applied as vaccine that is expected to induce a systemic immune response against unmodified residual tumor cells that otherwise lead to metastases. In mouse models using syngenic M5 or B16 melanoma cells (see report group Max L. Birnstiel) the vaccination with gene-modified tumor cells prevented the development of tumor in the prophylactic setting (vaccination before challenge with unmodified tumor cells) or the therapeutic setting (vaccination of animals bearing small tumors).

In early 1993, our Tumor Vaccine group at Bender started the development of an autologous, irradiated IL-2 modified melanoma vaccine. A adenovirus/transferrin-polylysine/DNA complexes were selected for the
transfection step. Protocols for the preparation of the protein conjugates were optimized. An IL-2 plasmid appropriate for GMP production was constructed by Dr. Gotthold Schaffner (IMF). With the help of Dr. Matt Cotten, a psoralen/UV-inactivated E4 defective adenovirus dII1014 was selected. Melanoma cell cultures were generated following protocols provided by Dr. Susanne Schreiber. Transfection of cultures (preferably with low passage numbers, <10 passages) resulted in high interleukin-2 expression levels between 1000 and 190000 units IL-2. The use of endotoxin-free DNA was found to be essential to avoid toxicity upon adenovirus-mediated gene transfer (Cotten et al., 1994).

![Graph showing gene transfer to murine B16 melanoma cells](image)

**Fig. 8:** Gene transfer to murine B16 melanoma cells (example shown: mGM-CSF expression) or human melanoma cells (NIL-2 expression) using synthetic peptide complexes.

For future tumor vaccine developments, we are evaluating peptide-transfection complexes (see above, chapter 1) as an alternative to the use of adenovirus complexes. Murine B16 melanoma cells and primary human melanoma cells were transfected efficiently using the peptide method, resulting in high levels of the cytokines GM-CSF and IL-2 secreted by the cells transfected with the corresponding genes (Fig.8).

**Publications during the year**


**Other references**


Rearrangement Genetic Changes in Tumorigenesis: Positional Cloning towards Tumor Preventing Genes

INTRODUCTION

Successful identification and molecular cloning of tumor- and tissue-associated genes may be achieved through knowledge of their genomic location. Often a candidate gene can be pinpointed owing to specific chromosomal rearrangements in a disease genotype. Once the location of such a gene is determined cytogenetically, marker DNA probes sufficiently close to the locus of interest may be used to reach it with positional cloning strategies.

In the past few years, we have focused our interest on the short arm of the human chromosome 1 (1p). Its distal region displays highly significant chromosomal aberrations, such as deletions and translocations, in a wide variety of human cancers including neuroblastoma and hepatoma. As a generally accepted hypothesis, the chromosomal rearrangements point towards the location of a putative tumor suppressor gene. In support of this hypothesis, the transfer of normal chromosome 1p material to neuroblastoma tumor cells is reported to suppress the malignant phenotype of the cells.

We have previously initiated a stepwise strategy to analyze the 1pter-p54 region in detail. This strategy consists mainly of two major parts: i) a detailed analysis of tumor-specific DNA rearrangements and ii) the detection, cloning, and characterization of the genes contained therein. The DNA analysis involved the detection of allelic deletions in tumor genomes with polymorphic microclone probes, the determination of a consensus deletion taking human neuroblastoma as a model, and the generation of a physical map using pulsed field gel electrophoresis (PFGE). Loss-of-heterozygosity analyses performed on neuroblastoma tumors revealed a consensus deletion of approximately 10 Megabase pairs (Mbp) in a subregion of the 1p36 band. We could, however, not assign a sufficient number of DNA markers to this consensus deletion; hence the identification and cloning of the gene loci contained could at this stage not be achieved as quickly and effectively as desired. We have therefore supplemented our stepwise strategy by isolating yeast artificial chromosome (YAC) clones and P1 phage clones specific for 1p36. Using this long-range cloning approach, we aim at saturating the region of interest with cloned DNA fragments. Newly established technical procedures shall be instrumental in mapping the clones by fluorescent hybridization (FISH) to tumor genomes and in directly screening cDNA libraries for corresponding genes.

While searching for genetic elements in the neuroblastoma consensus deletion, we have previously identified five Cpg island-associated genes, two of which we could map to the neuroblastoma consensus deletion. One of the encoded genes, HEIR1, displayed expression in normal adrenal medulla but not in neuroblastoma-derived cell lines. This gene was therefore subjected to a candidate gene approach in order to evaluate its potential function as a tumor preventing gene.

Long-Range YAC and P1 Cloning Using 1p36-Specific Microclones

Christoph Lengauer, Christoph Barnas, Patrick Onyango, Elke Kleiner, Traudl Henn, and Andreas Weit, in collaboration with Ramath Elaswarapu (HGMP Resource Centre, Harrow, UK.), Fiona Francis and Hans Lehrach (ICRF London, UK.)

We have generated approximately 3500 phage clones by means of microdissection and microcloning of distal chromosome 1p fragments in order to provide a high density of DNA markers for this genomic region. These clones have been generated using our standard protocol (Weit, 1993a) that yields insert sizes of 2-3 kbp mean size. Hence most of the clones are sufficiently large to be used as 32P-labeled Southern hybridization probes. Of these clones, approximately one-third have been identified as single-copy fragments using plaque hybridization with genomic DNA as a probe. 248 of the single-copy clones were mapped to date to the 1pter-p35 interval by means of somatic cell hybrid mapping. 211 of the 248 clones were localized to the 1pter-p36.12 interval, the others mapped proximal to 1p36.11-p36.3.

Of the clones located in the 1pter-p36.12 interval, 72 have thus far been used to isolate YAC and P1 clones. The technical approach involved the selection of pools of probes, each pool consisting of eight clones that generate hybridization patterns of equal intensity. Such pools of eight were then labeled with 32P-dCTP/dATP and hybridized to membranes containing DNA of gridded YAC or P1 phage clones. Screening of the YAC library initially yielded an unexpectedly high number of positive clones; however, the majority of these clones could be identified as false positives. The P1 clone library screening was more efficient, resulting in clearer hybridization signals and hardly any false positive signal. Eventually, 47 1p36-specific YACs and 14 P1 phage clones were identified. The location of these clones in 1p36 could be confirmed by means of fluorescence in situ hybridization (FISH). Using highly sensitive FISH analysis, 23 of the 47 YACs were found to hybridize both to 1p36 and to other genomic regions, indicating that these clones were chimeric (Fig. 1a). The other 24 YACs proved to be specific for 1p36 (Fig. 1c), although 14 of them showed cross-hybridization to the centromeric region of chromosome 1 (Fig. 1b).

Of 69 P1 phage clones tested to date, all but one mapped to 1p36 and were non-chimeric. Pulsed field gel electrophoresis of YAC clone DNA revealed an average insert size of 260 kbp, with a range from 80 to 680 kbp. The inserts of P1 phage clones displayed a fairly constant insert size of about 80 kbp. The isolated YAC clones cover about 6.7 Megabase pairs (Mbp), and the 59 P1 phages of this region span approximately 3.5 Mbp of DNA. As the YAC and P1 clones were isolated mainly with different microclone probes, they might not substantially overlap. The overall length of DNA included in the long clones may therefore comprise more than 9 Mbp. This would correspond to roughly 30% of the region being covered with clones. We may therefore stand a good chance to saturate 1p36 with long clones using all single-copy clones of the microclone libraries, provided YAC and P1 libraries with a high enough complexity are used.

Previous PFGE experiments employing 1p36-specific microclones have established a series of clusters comprising clones that are physically linked according to their partly identical hybridization pattern. Since we have been able to identify YAC and P1 clones with probes of the PFGE clusters, we can now link the location of several distinct long clones to 12 individual probe clusters. This provides the unique opportunity to combine the PFGE mapping data with the FISH analyses of the long clones. As a first important result from this, we may be able to establish a relative order of the PFGE clusters. This combination of analyses is expected to result in the saturation of 1p36 with an ordered array of long DNA clones.
**FISH Analysis of 1p36 Breakpoints in Tumour Cell Lines: Mapping Tool and Directed Search for Aberrant Genes**

Christoph Lengauer and Paula Stapleton

FISH analysis of chromosome 1p36 alterations in tumor derived cell lines was performed in order to reach two goals: i) to sublocalize YAC and P1 clones relative to the neuroblastoma consensus deletion, and ii) to identify long DNA clones that are located close to or even span the breakpoint of balanced translocations.

We were able to more precisely map YAC and P1 clones by performing FISH to three different tumor-derived cell lines (Fig. 2). One neuroblastoma-derived cell line had already previously been characterized as having a t(1p36.11;7p14) breakpoint; it thus served as a useful tool to identify all those clones localized proximal of the consensus deletion. Indeed, none of the clones selected to date hybridized proximal to the deletion breakpoint in this cell line. Another neuroblastoma-derived cell line was found to have an interstitial deletion encompassing the very distal 1p36.32 band but leaving the telomeric and 1p36.33 region heterozygous. By FISH on chromosomes of this cell line we could thus delineate all clones localized distal of the consensus deletion (Fig. 2). The third cell line was derived from a primitive neuroectodermal tumor (PNET), but displayed a balanced translocation involving the 1p36 band (Fig. 3a). The chromosomes of this cell line served to subdivide the YAC and P1 clones into a proximal and a distal consensus deletion group. Figure 2 summarizes the FISH data on the cell lines obtained to date and illustrates that most of the long clones mapped to the proximal part of the consensus deletion.

Since the PNET cell line shown in figure 3 displays a translocation which is balanced, we argued that the identification of the breakpoint might lead us directly to a gene whose defect plays a role in the malignant transformation. Hence we are using Cosmid, YAC and P1 probes to pinpoint the breakpoint between two loci and, if possible, to localize it in a particular YAC or P1 clone. To date, we have been able to map the translocation breakpoint between the PAX7 gene and the D1596 locus (Fig. 3b). Further characterization of this and other breakpoints will lead us to a better definition of the region containing the putative neuroblastoma tumor suppressor gene.
HEIR1/id3: A Helix-Loop-Helix Gene Involved in Development and Neuroblastoma Tumorigenesis?

Wilfried Eilmeier

A recently identified protein dimerization motif, the helix-loop-helix (HLH) domain, has been shown to be present in several transcriptional regulators. HLH transcription factors are involved in various developmental pathways. In Drosophila, HLH proteins encoded by the genes of the achaete-scute complex are involved in the development of the peripheral nervous system. In vertebrates, HLH proteins play a role in the expression of immunoglobulin genes in B-cells, in the expression of insulin in pancreatic β-cells and in different developmental pathways such as muscle differentiation and neurogenesis.

Homo- or hetero-dimers of HLH proteins that contain a cluster of basic amino acids adjacent to the HLH domain (basicHLH, dHLH) are able to bind DNA at a consensus target site. DNA binding of these bHLH proteins is regulated by dominant-negative HLH proteins (dnHLH) which lack the basic domain responsible for DNA binding. The first mammalian dnHLH protein identified was Id1 (formerly designated Iδ). It has been shown that Id1 can abolish DNA binding of the bHLH proteins MyoD and E12/47 in vitro. Furthermore, the in vitro binding ability of Id1 to E12/47 reflects an in vivo association of these two HLH proteins. In addition, it has been shown that over-expression of the Id1 protein in myoblasts and in myeloid cells impairs their ability to differentiate and that revertants of myoblasts, which are able to differentiate, had lost their expression of the Id1 protein. These observations have led to the model that Id1 acts as an inhibitor of differentiation by sequestering bHLH proteins necessary for differentiation from DNA binding (for review about HLH genes see Kadesch, 1993).

Recently, two mammalian Id-like dnHLH proteins have been identified. One of these genes is designated Id2; the third gene different names exist in the mouse and the human: the mouse gene was designated HLH462 (Christl et al., 1991) whereas for the human HEIR1 was chosen for reasons explained by Eilmeier et al. (1992). We now propose that the gene should be designated Id3 both in mouse and human for reasons of clarity. It has been shown that the two proteins Id2 and Id3, like Id1, can abolish DNA binding of bHLH proteins in vitro and that Id3 is an intermediate early gene. In addition, we observed that both murine and human Id3 show a mutual exclusive expression with respect to N-myc in human neuroblastoma tumor cell lines and in the developing murine forebrain suggesting a cross-talk between these two genes (Eilmeier et al., 1992).

In order to learn more about Id3, we have focused recent research on three major aspects: (1) Id3 in neuroblastoma, (2) expression of Id3 during embryonic development and (3) identification of proteins which interact with Id3.

Id3 in neuroblastoma

The experimental approach is directed towards understanding the function of Id3 concerning its putative role in neuroblastoma tumorigenesis. We are sequencing the promoter and the coding region of Id3 in primary neuroblastoma tumors and in neuroblastoma cell lines to analyze whether mutations in the Id3 gene are correlated with the occurrence of this cancer. To date no obvious sequence rearrangement has been detected in the DNA of 13 different tumors and tumor-derived cell lines.

For a functional analysis we are transfecting an inducible expression construct containing the Id3 gene into neuroblastoma tumor cell lines to establish stably transfected cell lines. These cell lines will be used for tumorigenicity studies in nude mice. The results of these experiments are expected to give an answer to the question of a possible involvement of HEIR1 in neuroblastoma tumorigenesis.

Expression of Id3 during embryonic development

The experimental approach addresses the role of Id3 during development and differentiation. By means of RNA in situ hybridization we have studied the expression pattern of Id3 in murine embryos. We observed that the expression pattern of Id3 is similar to that described for the Id1 gene in most tissues of the developing embryo. Id3 is expressed in the developing brain including the pituitary gland, in the spinal cord, in the olfactory epithelium, in the branchial arches, in the cardiac cushions of the developing heart, in the lungs, in the outer lining of the intestine, in the sclerome and in the limb buds. Thus, the expression pattern of Id3 in these tissues partially overlaps with Id1 expression. We, however, found that Id3 is expressed during tooth development, in distinct areas of the embryonic midbrain and in the collecting system of the developing metanephros (Fig. 4). Expression of the Id1 gene in these regions is not reported to date.

We could furthermore show with these experiments that our initial observation of mutually exclusive expression of Id3 and N-myc can be extended to other structures of the embryo between gestational stages E11.5-E15.5. It is worth, however, noting that some tissues co-express both genes within this period and at earlier stages (E9.5-10.5).

The results of the RNA in situ analysis suggest that Id3 may have unique functions during tooth and kidney development, whereas in other tissues of the developing embryo the two dnHLH genes (Id3 and Id1) may be functionally redundant.

Identification of Id3-binding proteins

One of our experimental approaches focuses on the identification of proteins which interact with Id3 via the HLH domain. The technical procedure makes use of the "interaction cloning" strategy. These cloning experiments were performed in the laboratory of Rene Bernards (Netherlands Cancer Institute, Amsterdam). Interaction cloning represents a method by which an expression cDNA library is screened with labeled protein to detect cloned targets by protein-protein interactions. After screening such a library from embryonic rat spinal cord (kindly provided by Tom Jessel, Howard Hughes Med. Inst., New York), we could identify three proteins which bind in vitro to Id3. Characterization of these proteins revealed that they belonged to the family of ubiquitously expressed bHLH proteins. Future work will focus on the significance of these interactions.

Fig. 4: HLH462 expression in the developing brain. Parasagittal section through brains of embryos at gestational day 13.5. A, C, E, G: bright-field views; B, D, F, H: corresponding dark-field views. (A and B) Forebrain: expression of HLH462 is restricted to the hippocampal region (H). (C and D) Midbrain: HLH462 is expressed in the ventral part of the midbrain. (E and F) Hindbrain: HLH462 hybridization shows expression in the metanephros (met) and in the myelencephalon (mye). (G and H) Pituitary gland. HLH462 is expressed in the pars intermedia (pi).
Studies Directed Towards the Detection of Asynchronous Replication in 1p36
Paula Stapleton

As an alternative approach to identifying the neuroblastoma tumor suppressor gene, we have been pursuing the association between replication timing, imprinted gene regions and chromosome 1p36. In the last 20 years of neuroblastoma research, no homozygous rearrangements have been observed in the region of interest in 1p36. The loss of heterozygosity studies in our lab and others indicate allelic loss at 1p38.2 - 36.1. In all cases the second allele appears to remain intact. It has long been postulated that the remaining allele contains subtle sequence alterations that render it non-functional. It is also possible, however, that this remaining allele has been subjected to epigenetic modifications that alter its expression. Recent work by Howard Cedar and colleagues has pioneered a method using fluorescent in situ hybridization (FISH) to study the replication timing of known imprinted genes in murine and human genomes (Klipsberg et al., 1993). FISH to interphase nuclei was used to score replication patterns of probes to various loci and it was found that known imprinted genes and their human homologues lie in domains that replicate asynchronously compared to other loci in the genome. This work suggests that asynchronous replication represents a general characteristic of imprinted gene regions in both embryonic and somatic cells. In view of these results and the technique used to determine asynchronous replication we were interested in using this method to analyze loci in 1p36. Probes specific to 1p36 (Id3, C52, PAX7, C1-45), as well as a positive control (IgH), and a number of other Cosmid probes scattered throughout the genome, were analyzed. Figure 5 shows a representative example of a hybridization to G1 and S-phase nuclei. Preliminary results indicate that Id3 and C52 lie in a region that is asynchronously replicating. Further indications for an imprinted locus on 1p were recently published in a study demonstrating maternal specific allele loss in 13 out of 15 neuroblastoma tumors examined (Caron et al., 1993). When taken together these data support the idea that 1p36 contains a genomically imprinted locus; however, it will be important in future experiments to set up a system that allows us to determine whether the asynchronous replication we observed is allele specific.

Fig. 5: FISH analysis of replication timing at the HER1 locus. Cosmid DNA containing the gene sequence was labeled with bio-11-dUTP and detected with FITC avidin after hybridization to interphase nuclei. Specific labeling of S phase nuclei has been achieved by BioUl incorporation followed by detection with rhodamine conjugated anti-BioUl antibody (dark grey label on right nucleus). Note that the two single-dot signals in the left nucleus correspond to non-replicated alleles. The right S phase nucleus displays both a single-dot and a double-dot signal, the latter representing a replicated allele.

Localization of Chromosome 9p Rearrangements Relative to the PAX5 Locus: Is PAX5 involved in B-cell Malignancies?

Loss or rearrangement of chromosome 9p has been observed in a number of malignancies including lung tumors (both non-small cell lung carcinoma and malignant mesothelioma) and lymphomas of B-cell origin and appears to be a significant feature in these particular tumors. In this context is is of interest that we recently have mapped PAX5 to chromosome 9p13 (Stapleton et al., 1993) and that PAX5 is thought to have an essential function in normal B cell differentiation (see this year's research report of P. Urbánek et al., group M. Bussinger). As other members of the PAX gene family have been associated with human diseases (PAX3 with Waardenburg’s Syndrome and PAX6 with Aniridia; reviewed in Gruss and Walther, 1992), we thought to determine whether PAX5 plays a role in B-cell malignancies showing alterations in 9p. To achieve this aim, five cell lines derived from B-cell malignancies, both B-cell non-Hodgkin’s lymphoma (B-NHL) and B-cell precursor acute lymphocytic leukaemias (BCP-ALL), were studied. All cell lines studied exhibit alterations in the chromosome region 9p11-13 (M. Dyer, personal communication). To begin our study, we are using FISH to analyze the breakpoints in chromosome 9p with respect to PAX5. The results are outlined in Table 1, however, as yet it is not clear whether PAX5 plays a role in B-cell malignancies.

<table>
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<tr>
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<td>proximal</td>
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* EBV transformed cell lines derived from B-cell malignancies
B-NHL: B-cell non-Hodgkin’s lymphoma
BCP-ALL: B-cell precursor acute lymphocytic leukaemia

Table 1: Analysis of Breakpoints in 9p Relative to PAX5 in B-cell Malignancies
Nuclear Oncoproteins and Transcription Factors in Hematopoietic Cell Differentiation

INTRODUCTION

Differentiation of hematopoietic cells involves the highly ordered and controlled proliferation of immature progenitor cells and their commitment and differentiation into fully mature cells of various lineages. This process is tightly controlled to ensure that all cell-types of the hematopoietic system are produced in a balanced fashion according to the needs of the organism. In leukemias such normal control mechanisms are bypassed leading to a massive and uncontrolled outgrowth of largely immature hematopoietic cells which cause the disease.

The focus of research in this laboratory is to study genes and protein factors which serve key roles in hematopoietic cell differentiation and which upon oncogenic activation cause leukemia. So far these studies have been mainly performed in an experimental model system using oncogene-transformed bone marrow cells of chicken. In this system, oncogenic retroviruses are used to infect chicken bone marrow cells in tissue culture; then various aspects of hematopoietic cell differentiation and leukemogenesis can be studied in vitro under well-defined experimental conditions. A unique advantage offered by the chicken system is that such oncogene-transformed cells retain their capacity to undergo apparently normal terminal differentiation in vitro, once the transforming oncogene is experimentally "switched off".

Using this approach, we have over the past several years extensively studied the impact of the erbA/T3 receptor molecules and of the erbA-related receptors RAR and RXR (for all-trans retinoic acid [RA] and 9-cis RA, respectively) on erythroid cell differentiation. Additionally, we have taken advantage of our in vitro red cell differentiation system to investigate differential functional properties of the GATA-1,-2 and -3 transcription factors in red cell development.

In an effort to identify an oncogene which would transform an early, potentially multipotent hematopoietic progenitor cell, the v-rel oncogene was employed. A conditional hormone-inducible v-rel estrogen receptor fusion (v-relER) was constructed and the differentiation potential of v-relER transformed chicken bone marrow cells is currently being studied.

Since several lines of evidence suggest that the retinoblastoma gene product Rb might serve an important function during hematopoiesis, we have now cloned and characterized the chicken homologue of Rb. Chicken Rb-specific antibodies were also prepared and will be used to study Rb expression and function in our in vitro differentiation systems.

Since virus-mediated gene transfer appeared to be the only efficient means to introduce and express exogenous genes in primary hematopoietic cells, alternative techniques were tested. The conditions developed should be of particular interest for gene therapy in hematopoietic progenitor and/or stem cells.

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1. Transcriptional regulators of red cell differentiation

1.1. erbA, RXR and RAR

(Petr Bartunek)

Previous studies established that thyroid hormone (T3) and retinoic acid (RA), by activating the c-erbA/T3 receptor and RA receptor, respectively, accelerate red cell differentiation (Zenke et al., 1980; Schroeder et al., 1992). Furthermore, both the c-erbA/T3 and the RA receptors require the auxiliary protein factor RXR for sequence-specific binding to their cognate DNA response elements (see Stunnenberg, 1992, for a review). Since RXR itself represents a receptor for the RA isoform 9-cis RA, we have now tested the impact of 9-cis RA and of a series of synthetic RA derivatives on red cell differentiation (in collaboration with A. Levin, Hoffmann-La Roche, Nutley, USA and P. Chambon, Faculté de Médecine, Strasbourg, France). Both normal, untransformed stem cell factor (SCF)-dependent red cell progenitors (see Research Report Hartmut Beug) and erythroblast clones transformed by conditional, temperature-sensitive ts oncogenes were used for these studies. v-erbA and r12-v-erbA transformed erythroblasts were also employed.

As shown before, both v-erbA and the hypertransforming v-erbA variant r12-v-erbA efficiently block red cell differentiation (Zenke et al., 1988, 1990; Damm et al., 1987). While v-erbA erythroblasts can be induced to differentiate by treatment with all-trans RA (atRA), r12-v-erbA erythroblasts cannot (Schoeder et al., 1992; Baretino et al., 1993). The reason for this is that r12-v-erbA efficiently sequesters RXR, an activity which is lost in v-erbA (Baretino et al., 1993; Fig. 1). We are presently evaluating the specific effects of a series of synthetic RA derivatives and potential RXR-specific ligands on the differentiation potential of erbA transformed erythroblasts. We expect that these studies shall eventually allow us to dissociate RAR- and RXR-specific effects on red cell differentiation.

1.2. erbA target genes

(Karoline Briegel and Gabi Stengel)

In 1993 we continued our efforts to isolate new target genes of c- and v-erbA in order to identify new regulators of erythroid differentiation. Both v-erbA as well as an overexpressed c-erbA/TR3 receptor (in the absence of thyroid hormone) efficiently block red cell differentiation, whereas a hormone-activated c-erbA/TR3 receptor enhances terminal differentiation of these cells (Zenke et al., 1990). Since the expression pattern of the three known red cell-specific erbA target genes (carbonic anhydrase II [CAII], the anion transporter band3 and aminoethylamine acid synthase [ALA-S]) can explain only part of the erbA phenotypes (Zenke et al., 1988, Fuertenberg et al., 1992), we speculate that other targets must exist which should play a more decisive role in erythroid development.

By using conventional differential cDNA cloning, we have cloned several erbA-regulated genes whose functions are currently being investigated. More recently, PCR-based differential display was also employed and the analysis of the genes obtained is in progress.

1.3. GATA transcription factors

(Karoline Briegel in collaboration with D. Engel, Northwestern University, Evanston/Chicago, USA)

The GATA factors are a family of Zn-finger transcriptional regulatory proteins which display a highly conserved tissue-specific and temporal expression pattern in all eukaryotes. Interestingly, three members of this family, GATA-1, -2 and -3, are coexpressed at different and changing ratios during normal red blood cell differentiation. Whereas GATA-1 is known to represent a major regulator of erythropoiesis, the biological functions of GATA-2 and -3 in this lineage had remained obscure. The highly conserved expression pattern throughout erythroid development suggested to us that the relative abundance of the GATA factors (which ostensibly share the same DNA binding site) with respect to one another is developmentally controlled and might play a role in influencing the decision of an erythroid progenitor cell to undergo either self-renewal or terminal differentiation.

In our previous studies we addressed this question by ectopically expressing GATA-1, -2 and -3 and conditional versions thereof (GATA/estrogen receptor fusion proteins) in primary chicken erythroid progenitor cells where both cell proliferation and differentiation can be experimentally modulated (Briegel et al., 1993). It was found that GATA-2 has an important function in erythroid progenitor cells, since such cells remained immature and retained their self-renewal capacity (Fig. 2). We conclude therefore that developmentally controlled down-regulation of GATA-2 appears to be a prerequisite for normal terminal differentiation of red cell progenitors (Briegel et al., 1993). Conversely, GATA-1 exerts a differentiation-promoting activity, because an ectopic GATA-1/ER fusion protein drastically accelerates terminal differentiation of erythroblasts in a hormone-dependent manner.

While GATA-2 expression is largely regulated at the mRNA level, GATA-1 activity is modulated posttranslationally, which is a subject of current studies. Finally, GATA-3 has no obvious effect on erythroid differentiation in our assay system (Briegel et al., 1993), and hence might be more likely a regulator of T lymphocyte development where it is predominantly expressed.
Several lines of evidence suggest that Rb might act as a binary switch important for both cell proliferation and terminal differentiation. The most compelling evidence for an important role of Rb in normal differentiation comes from the analysis of gene inactivation experiments in mice (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). While these experiments demonstrate that Rb is apparently dispensable during the early phase of embryonic development, they point to an important function of Rb during hematopoiesis. As a first step to study Rb function in normal hematopoiesis and to investigate whether the normal Rb expression pattern is altered in oncogene-transformed cells of chicken, we have cloned the chicken Rb (chrB)-specific cDNA and provide an initial characterization of the protein product it encodes (Boehmelt et al., 1994).

Chicken Rb was found to be highly homologous to human, mouse and Xenopus Rb in primary amino acid sequence, underlining its conserved function in cell cycle control in higher eukaryotes. The highest level of homology was found within the Rb "pocket domains" and at the Rb C-terminus, whereas the extreme N-terminus of chrB is divergent. Transcription of chicken Rb initiates at multiple start sites within a DNA segment which represents a CpG island. Chicken Rb is transcribed as a 4.7 kb mRNA species and encodes a 104 kDa protein which is therefore slightly smaller than the mammalian Rb proteins. The Rb cDNA together with chicken Rb-specific antibodies now provide the reagents for testing whether a number of retinal oncoproteins of chicken affect Rb gene expression in transformed cells and/or modulate the Rb protein activity through direct protein-protein interactions. Such studies should elucidate whether and how altered chicken Rb expression and/or activity contributes to the altered growth and differentiation properties of such oncogene-transformed cells.

4. Gene transfer into human and chicken hematopoietic progenitor cells

(Ad collaboration with M. Cotten, E. Wagner and M.L. Bimsstel, I.M.P., Vienna)

So far, efficient gene transfer into primary hematopoietic cells employs mainly viral vectors and stable integration into the host cell DNA. In an effort to explore alternative techniques, gene transfer by receptor-mediated endocytosis via the transferrin receptor (Wagner et al., 1990; Zennke et al., 1990; Cotten et al., 1990; see also Research Reports of M. Cotten, E. Wagner and M.L. Bimsstel) was found to be highly efficient. Conditions were developed for gene transfer into oncogene-transformed and untransformed hematopoietic progenitor cells of chicken and also for CD34+ human peripheral blood progenitor/stem cells. Expression of the genes introduced was transient and found to vary considerably depending on the cell-type analyzed.

These studies establish that receptor-mediated gene transfer can be used for primary hematopoietic progenitor/stem cells which are notoriously difficult to transfect by standard techniques.

Publications during the year


2. The v-rel oncprotein transforms a pluripotent hematopoietic progenitor cell

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

We previously described the biological and biochemical activities of a chimeric protein containing v-rel fused to the hormone-binding domain of the human estrogen receptor. This v-rel/ER protein causes estrogen-dependent, but otherwise unaltered v-rel-specific transformation of avian fibroblasts and bone marrow cells (Boehmelt et al., 1992). Individual v-rel/ER transformed bone marrow cell clones were isolated and expanded in liquid culture. We have now analyzed their differentiation potential following inactivation of v-rel/ER activity by administration of the estrogen antagonist ICI 164,384. Experimental conditions were established which support differentiation of a given v-rel/ER cell clone into at least 2 distinct hematopoietic cell types. This finding demonstrates that v-rel/ER (and presumably also the unmodified v-rel) transforms an early pluripotent hematopoietic progenitor cell. Characterization of the various differentiated cells obtained is in progress.

3. Structure and expression of the chicken retinoblastoma gene

(Guido Boehmelt, Robert Kurzbauer, Georg Mellitzer and Jaime Madruga in collaboration with E. Ulrich and A. Bird, University of Edinburgh, Edinburgh, UK)

The protein product of the retinoblastoma susceptibility gene Rb is one of the fundamental regulators involved in cell cycle control in higher eukaryotes. Initially identified as a tumor suppressor gene (anti-oncogene) in inherited retinoblastoma in man, functional inactivation of Rb by deletion and/or point mutation has subsequently been found in a variety of other human tumors, and also occurs through its association with the oncogene products of DNA tumor viruses (see Weinberg, 1991 for a review).

Other references


The animal house provides technical support to the scientists and at present holds various in- and outbred, as well as hybrid strains of mice, random-bred rabbits and out-bred chickens. At the end of 1992, we took over the chicken facility within the University Biocenter. This is now well-established and provides us with fertilized eggs and more chickens for experimental procedures.

Current production of animals bred in-house is as follows: approx. 400 mice/week, 70 chicken embryos/week and 10 chickens/week. For economic reasons, rabbits are purchased from external suppliers, as are some mice for short-term studies.

The animals listed above service the following studies: various mouse projects (see Reports of Erwin Wagner, Denise Barlow, Meinrad Busslinger, Andreas Weil and Max Birmstiel) and chicken leukemia studies (see Reports of Hartmut Beug and Martin Zenke). Due to the increased number of experimental mouse studies, an extra room has been built to accommodate three large filter cabinets for holding mice of a specified quality. In addition, a new micro-injection laboratory is being constructed and will be operational early next year.

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

Scientist
Anton Beyer
PhD student
Aron Bauer
Diploma student
Bianca Habermann

Introduction

Our main interest is the relationship between amino acid sequence and tertiary fold of globular proteins. Atomic coordinates from crystal structures can be used to construct a potential of mean force. Aron Bauer has developed new methods for constructing such a potential. Besides data from known crystal structures he also uses information from sequence data alone. Applying this new matrix method for constructing the potential, the quality of finding the right structure for a given sequence is significantly increased. This work will be described in detail below.

Improving the Calculation of Protein Stabilizing Interactions

Aron Bauer

Statistically derived potentials have become an important tool in understanding the link between protein sequence and structure. We are developing such potentials and look for ways to improve their performance. Although the number of available structures rises steadily, the hardest problem still is the lack of sufficient structural data on proteins. To overcome critical difficulties in the underlying statistics, so called smoothing procedures have to be devised.

In the past year we demonstrated that a smoothing method we have developed proves to be superior to comparable approaches (Bauer & Beyer, 1994). The Matrix Method is based on the use of mutation data matrices and does not require the optimization of parameters, which is a serious drawback in other approaches, since their value can be estimated after time consuming series of calculations only. The Matrix Method can therefore be easily applied to different kinds of potentials.

Previously we had focused on Cα-Cα pair interaction potentials only. The results obtained on several experiments demonstrated the need of terms accounting for solvation. We have shown that efficient solvation potentials can be derived from exactly the same statistics that is used for the inter-residue (pairwise) terms. This feature enables the combination of both potentials without the need for relative scaling. The addition of solvent terms enhances the performance of the mean force potentials significantly. From the obtained solvation potentials a hydrophobicity scale was derived. Comparison with other common scales yields good correlation.

Furthermore we are evaluating terms depending on local dihedral conformation, which are not covered by the current pair potential.

The quality of the potentials we use was assessed by a simple threading experiment. A more sophisticated method based on dynamic programming was devised and yields impressive results in revealing distant homologies or structural analogies in a sequence-structure matching approach. This method of structural database screening is the most important application developed. We have started to write an interface-driven easy-to-use computer program that includes our current knowledge and techniques and will be of general use for sequence analysis.

Another application of the statistically derived potentials is the generation and optimization of sequences for a given fold or tertiary structure. Proteins with no detectable sequence homology are sometimes found to have similar or almost identical folds. We want to estimate the sequence space accessible for a given protein structure, i.e. how many and how different sequences will fold into nearly the same 3-dimensional structure. Preliminary results suggest, that (according to the current set of potentials) the sequence space for a given structure is vast, and optimized sequences are easy to obtain. Comparisons with native sequences reveal sequence homologies slightly beneath the level of statistical significance (the twilight zone). This sheds a new light on standard methods of sequence database screening and might be of use in the development of improved scoring methods to assess sequence-structure matching significance.

Statistics and evaluation of correlation between residual temperature factor, variability of sequence, surface accessibility and residual empirical energy of proteins

Bianca Habermann

Protein sequences and three-dimensional structures reveal an amount of information other than only the fold of the chain. A multiple protein sequence alignment yields the frequency of amino acid exchange of a residue, the variability (Sander & Schneider, 1991, and references therein). Crystallographic structure determination provides the temperature factor (Debye-Waller factor) B (Petso & Ringe, 1984, and references therein). The temperature factor is a measure of distortion of atoms within the crystal and arises from the root-mean-square deviation of their displacement. It is taken from the protein structural data of the Brookhaven database (pdb-database). Once the three-dimensional structure of a protein is known, one can calculate the accessibility of the residues (Sander & Schneider, 1991). This is performed using the Lee and Richards definition of the accessible surface which is determined by calculating the contact points of the side chains with a probe of the solvent. The solvent probe is defined as a water molecule rolling around the protein surface, while remaining in contact with the protein molecule (Richards, 1992). The accessibility as well as the variability is included in the HSSP (Homology-derived Secondary Structure Prediction)-files by C. Sander et al. and is available via Internet (EMBL-server, Heidelberg) (Sippl, 1990). The Potentials of Mean Force (pmf) derived energy is another factor used for describing protein structures. It is based upon statistical evaluation of Cα-Cα distances within a number of chosen non-homologous protein chains, tracing spatial characteristics of protein folds (Sippl, 1990; Bauer & Beyer, 1994). A residual energy is derived by calculating Cα-Cα energies of a residue with its surrounding, based on the Potentials of Mean Force. Since the most significant contributions of stabilization are due to short range interactions, a cutoff of 8Å was additionally chosen to calculate residual energies. Another important factor for stabilizing a protein structure is the interaction with the solvent. A pseudo solvation energy, which is also based on statistical measures, was calculated, using 'holes' within the packing density of Cα atoms in the surrounding of a Cα, whereas the holes are represented by the difference between the maximum packing density and the density measured within a distance d. These pseudo solvation energies were calculated with and without a cutoff of 8Å. All the Potentials of Mean Force derived energies were provided by Aron Bauer.

These factors mentioned above were chosen to create datatables for 167 selected, non-homologous protein chains. The factors were statistically analyzed in terms of their distribution, and their correlation to each other. Global analysis as well as separate analysis of the 20 different amino acid types was performed. The aim of this work was to get more information on the statistical behavior of protein folds in terms of their variability, mobility, the inside-outside preference of their residues and their stability. Global analysis reveals an even distribution of the factors and lacks significant correlations between the chosen factors. Statistical analysis of the different amino acid types shows more distinct distribution depending on the amino acid type, as well as better correlation. However, the distribution of the variables, in residue specific terms, shows only slight differences concerning the mean value of the variables. Furthermore, the standard deviation of the variables is high, thus no profitable use may be seen in the difference of pmf distributions. The correlation of the variables is also not significant enough to make reliable predictions of their behavior. It is noteworthy to mention the lacking correlation between the accessibility and the variability of the temperature factor, displaying the mobility of a residue, and the accessibility is not really significant, contrary to expectations. An evaluation of the temperature factor...
as a measure for thermal mobility is currently done by comparing molecular dynamics data with the temperature factors derived from crystal data for the small protein ubiquitin. Molecular dynamics data were provided by Heilfried Schreiber.

The conclusion that has to be drawn from this statistical work is the fact, that the distributions are rather broad and there appears to be only a weak correlation between the different factors investigated.

Publications during the year


Other references


Introduction

The Service Department offers many rapid services to members of the institute. The most time consuming ones are DNA sequencing and oligonucleotide synthesis. A small but substantial part of our work is provided to institutions outside the I.M.P.: we sequenced a few DNA samples and synthesized about 150 oligonucleotides for university institutes in Vienna and Salzburg. Another project was the construction of a high copy number vector carrying the tetracyclin resistance gene for the "Tumor Vaccine Project" (Benderl/I.M.P., Vienna). Projects from I.M.P. scientists nevertheless always have the first priority.

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

The high quality of the cell culture media and numerous solutions provided by our Media Kitchen is a prerequisite for the continuity and reliability of cell culture and for most of the other work done at the institute. We also prepare many selected reagents, such as DNA molecular weight markers, enzymes and transformation competent E. coli strains, and keep stock of cloning vectors, primers etc.

Oligonucleotide Synthesis

As in the previous year, we synthesized about 1800 oligonucleotides in 1993 (Fig. 1). Many of them were used as PCR primers, and others were used for sequencing.

We have seen in the last years that the demand for oligonucleotides is heavier in certain periods of the year (spring/early summer and fall/early winter, Fig. 2). The demand for synthesizing oligonucleotides became so heavy in these periods that it was necessary to work Saturdays and Sundays in order to produce them all. For this reason we bought an additional oligonucleotide synthesizer (PERKIN-ELMER/ APPLIED BIOSYSTEMS 394) in late fall.

For quantitative and in situ PCR, we have made some first attempts with 6-Fam (P-E/ APPLIED BIOSYSTEMS) fluorescently labeled oligonucleotides.

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

It became clear that we could not increase the total sequencing capacity by another factor of 2 with the same amount of people, as we did last year (Fig. 3). Nevertheless, the total number of reactions done this year was again higher. About 2900 DNA samples were sequenced the conventional way using SEQUENASE (USB) and the sequense protocol with radioactive 32P-dATP. We would prefer to use 32P-dATP because of its lower radiation. Unfortunately it is not yet possible because of legal proscriptions.

The same number (2900) of samples was sequenced using TAA DNA polymerase and fluorescently labelled dyeoxy nucleotides (P-E/APPLIED BIOSYSTEMS). The analyses were performed on the automated sequencer (P-E/ APPLIED BIOSYSTEMS 373 A), which gave very reliable results even with difficult DNA's, such as large DNA's (>20 kbp) and PCR products. The dye dyeoxy technique has also advantages in DNA regions with high GC content. "Strong stops" are not visible because there is no light emission when the polymerase falls off without incorporating the fluorescently labelled dyeoxy nucleotide. Nevertheless, there are still many unsolved sequencing problems that we are continuing to work on!

Publications during the year


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I.M.P. Scientific Report 1993

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Diploma- and PhD-Theses of I.M.P. Students in 1993

Gabriele Bergers (Group Meinrad Busslinger)
Doctorate Thesis: "Identification and characterization of the two Fos-regulated genes fra-1 and fit-1", submitted to the University of Vienna.

Guido Boehmelt (Group Martin Zenke)
Doctorate Thesis: "A conditional v-rel oncprotein as a tool to study transformation and differentiation of multipotent hematopoietic progenitor cells", submitted to the University of Vienna.

Friedemann Kiefer (Group Erwin Wagner)

Anita Kobnra (Group Andreas Weith)
Diploma Thesis: "Structural Analysis of a Genetic Element Located in the Neuroblastoma Consensus Deletion", submitted to the University of Vienna.

Georg Mellitzer (Group Martin Zenke)
Diploma Thesis: "Das konditionelle v-relER Oncoprotein: Spezifische Auswirkungen in lymphoiden und erythropoi- den Zellen", submitted to the University of Vienna.

Reinhard J. Stöger (Group Denise Barlow)
Doctorate Thesis: "Positional cloning of an imprinted gene at the T-associated maternal effect (Tme) locus in Mus musculus", submitted to the University of Vienna.

Awards Granted in 1993

Max L. Birnstiel:
Full member of the Austrian Academy of Sciences (Vienna).

Agi Grigoriadis:
"Procter & Gamble Young Investigator Award" for the American Society of Bone and Mineral Research (Sept. 1993, Tampa, Florida).

Ernst Reichmann:
"Prize for Interdisciplinary Cooperation", donated by the "GSF - Forschungszentrum für Umwelt und Gesundheit" (Nov. 1993, Munich).

Michael Schlossmacher:

Erwin Wagner:

Zbynko Kozmik:
"Prize for Science", donated by Hlavka's Foundation and the Czech Academy of Sciences (Nov. 1993, Prague).
I.M.P. Seminar List 1993

JANUARY

07.01.93
JEFFREY WILLIAMS (ICRF, Herts)
"Interactions between the cyclic AMP dependent protein kinase and the DIF signal transduction pathways regulate Dictyostelium morphogenesis"
Host: Denise Barlow

21.01.93
HUGH PELHAM (MRC, Cambridge)
"Protein retention in the endoplasmic reticulum: sorting, receptors and receptor sorting"
Host: Meinrad Busslinger

28.01.93
WALTER BIRCHMEIER (Univ. of Essen)
"Cadherins and motility factors in the control of epithelial differentiation and invasiveness"
Host: Ernst Rechmann

FEBRUARY

04.02.93
CHRISTO GORIDIS (INSERM, Marseille)
"Neural cell adhesion molecules: versatility of function and regulation of expression"
Host: Meinrad Busslinger

12.02.93
HERBERT FLEISCH (University of Bern)
"Bone formation and resorption: physiology, pathology and pharmacology"
Host: Erwin Wagner

12.02.93
GÜNTHER SCHÜTZ (DKFZ, Heidelberg)
"Transgenic mice generated by pronuclear injection of yeast artificial chromosomes (YACs)"
Host: Hartmut Beug

16.02.93
STEFAN JENTSCH (F. Miescher Lab., Tübingen)
"Selective proteolysis by the ubiquitin system"
Host: Kim Nasmyth

17.02.93
PETER CARMELEIT (Univ. of Louvain)
"Gene inactivation of the fibrinolytic enzymes: u-PA, t-PA and PAI-1"
Host: Erwin Wagner

18.02.93
WERNER RISAU (MPI, Martinsried)
"Molecular mechanisms of vasculogenesis and angiogenesis"
Host: Hartmut Beug

MARCH

02.03.93
ROBERT ARKOWITZ (UCLA)
"Protein translocation and unfolding in E. coli"
Host: Max L. Birnstiel

04.03.93
HANS LEHRACH (ICRF, London)
"Molecular genetics of mammalian chromosomes"
Host: Denise Barlow

05.03.93
PETER ANGEL (Kernforschungszentrum Karlsruhe)
"Regulation of c-jun and Jun-dependent genes"
Host: Martin Hofmann

11.03.92
CESARE PESCHELE (Rome)
"Molecular and cellular basis of early human hematopoiesis"
Host: Thomas von Rüden

12.03.93
CHARLES LAIRD (FHCRC, Seattle)
"Genomic imprinting and DNA instability: lessons from the fragile-X syndrome"
Host: Kim Nasmyth

12.03.93
DAVID R. PARKINSON (NCI, Bethesda)
"Gene therapy of cancer: current concepts"
Hosts: Max L. Birnstiel and Georg Stingl

16.03.93
KARL-HEINZ PREISEGGER (NCI, Bethesda)
"Function of the multi-drug transporter and the regulation of the multi-drug resistance (mdr) genes"
Host: Kurt Zatloukal

18.03.9
ERIC WEINBERG (Univ. of Pennsylvania)
"Regional expression of achaete-scute homologue genes in the zebrafish embryonic brain"
Host: Max L. Birnstiel

29.03.93
MATTHIAS ERNST (Ludwig Institute, Melbourne)
"Generation of an hck transgene in situ in embryonic stem cells: Is hck part of a LIF signal transduction pathway?"
Host: Max L. Birnstiel

30.03.93
THOMAS BÜRGLIN (Harvard Med. School, Boston)
"Dissecting the role of homeobox genes in C. elegans development"
Host: Max L. Birnstiel

31.03.93
DIETMAR KUHL (Columbia Univ., New York)
"Identification of genes induced during learning and memory"
Host: Max L. Birnstiel
APRIL

01.04.93
CARL WU (NIH, Bethesda)
"Induction of heat shock gene expression in eukaryotes"
Host: Denise Barlow

02.04.93
FRIEDRICH PROPST (Ludwig Institute, London)
"Actions of the mos oncogene in transformed cells and transgenic mice"
Host: Max L. Birnstiel

08.04.93
ANTON BERNs (Netherlands Cancer Institute, Amsterdam)
"Mouse model systems to study the role of oncogenes and tumor suppressor genes in normal and malignant growth"
Host: Erwin Wagner

15.04.93
HARMUT LAND (ICRF, London)
"Cooperating oncogenes: function and links to cell cycle regulation"
Host: Hartmut Beug

20.04.93
ALBRECHT LUCKENBACH (Merck, Darmstadt)
"Immunotherapy of solid tumors"
Host: Bjorn Neumann

22.04.93
LEO KRETZNER (FHCRC, Seattle)
"The myc network of transcription factors"
Host: Hartmut Beug

28.04.93
ARI HELENius (Yale Univ.)
"Virus entry into cells: the assembly/disassembly paradox"
Host: Max Birnstiel

29.04.93
PETER PARKER (ICRF, London)
"Strategies in the control of the PKC pathway"
Host: Lisa Ballou

30.04.93
MANUELA BACCARINI (FGH, Hannover)
"Signaling transduction by cytosolic kinases in macrophage proliferation and differentiation"
Host: Max Birnstiel

30.04.93
JUAN BELMONTE (UCLA)
"Some insights into the molecular mechanisms underlying gastrulation and limb development in vertebrate embryos"
Host: Max Birnstiel

MAY

03.05.93
GERHARD CHRISTOFORI (UCSF)
"Molecular dissection of tumor progression and angiogenesis in a transgenic mouse model"
Host: Max Birnstiel

03.05.93
AMANDA FISHER (INSERM, Strasbourg)
"Lymphopoiesis and T-cell development"
Host: Max Birnstiel

04.05.93
LUKAS HUBER (EMBL)
"The role of rab GTP-binding proteins in post Golgi transport"
Host: Max Birnstiel

05.05.93
THOMAS JENUWEIN (UCSF)
"Molecular mechanism regulating locus control activity during mouse development"
Host: Max Birnstiel

05.05.93
PETER MATTHIAS (UCSF)
"The interface between a signal transduction pathway and the cell cycle machinery"
Host: Max Birnstiel

06.05.93
STEFFAN KRAUSS (ICRF, Oxford)
"Aspects of early brain patterning in zebrafish embryos"
Host: Meinrad Busslinger

13.05.93
MICHAEL RETH (MPI, Freiburg)
"Signal transduction from the B cell antigen receptor"
Host: Meinrad Busslinger

14.05.93
FRANZ THEURING (Schering, Berlin)
"Tissue-specific and developmental regulation of human p1a chimeric genes in transgenic mice"
Host: Marion Fung

21.05.93
CHRIS SANDER (EMBL, Heidelberg)
"From genome sequences to protein structure and function"
Host: Anton Beyer

25.05.93
PAOLO COMOGLO (Univ. of Turin)
"The hepatocyte growth factor/scatter factor receptor"
Host: Erwin Wagner
JUNE

15.06.93  THOMAS BLANKENSTEIN (Universitätsklinikum Steglitz, Berlin)
  "Mechanisms of cytokine induced tumor rejection"
  Host: Martin Zenke

16.06.93  ARISTIDES MOUSTAKAS (Whitehead Institute)
  "Functional interactions of the high-affinity receptors for TGFbeta"* 
  Host: Ernst Reimann

17.06.93  RUDI BALLING (MPI, Freiburg)
  "Mice with kinky tails: a genetic dissection of skeletal development"
  Host: Meinrad Busslinger

22.06.93  THOMAS W. STURGILL (Univ. of Virginia)
  "Upstream pathways for the activation of MAP kinase"*
  Host: Lisa Ballou

24.06.93  GERARD GROSVELD (Erasmus Univ., Rotterdam)
  "Molecular analysis of t(6;9) in acute nonlymphocytic leukemia"*
  Host: Hartmut Beug

JULY

01.07.93  ULF RAPP (NIH, Frederick)
  "Rat dependent signalling pathways: role in the control of growth and differentiation"
  Host: Tillman Schuster

08.07.93  SUSANNE OSANTO (AZL, Leiden)
  "Immunization of melanoma cancer patients with genetically modified melanoma cells producing IL-2"
  Host: Ernst Wagner

09.07.93  HAMILTON SMITH (John Hopkins)
  "Natural DNA transformation in bacteria"
  Host: Max L. Birnstiel

15.07.93  BRIAN JOHNSTON (SRI, California)
  "Approaches to gene targeting using recA protein"
  Host: Ernst Wagner

16.07.93  HENK STUNNENBERG (EMBL, Heidelberg)
  "Molecular mechanisms of transactivation by retinoic acid receptors"
  Host: Hartmut Beug

29.07.93  JEFF WAHL (The Salk Institute, San Diego)
  "How tumor suppressor gene function affects genetic stability by cell cycle control"
  Host: Andreas Weilh

AUGUST

17.08.93  OSKAR HAAS (St. Anna Kinderspital, Vienna)
  "Parental origin of chromosomes involved in the translocation t(9;22)"
  Host: Denise Barlow

SEPTEMBER

01.09.93  GORDON KELLER (Natl. Jewish Center, Denver)
  "Molecular aspects of hematopoietic development"
  Host: Erwin F. Wagner

02.09.93  HAROLD MOSE (Vanderbilt Univ., Nashville)
  "TGFbeta regulation of epithelial proliferation"
  Host: Hartmut Beug

09.09.93  MALCOLM PARKER (ICRF, London)
  "Structure and function of the estrogen receptor"
  Host: Hartmut Beug

16.09.93  JEAN BEGGS (Univ. of Edinburgh)
  "Nuclear pre-mRNA splicing in yeast"
  Host: Kim Nasmyth

20.09.93  HUBERT KOLB (Diabetes Institute, Univ. Düsseldorf)
  "Immunopathogenesis of type I diabetes"
  Host: Erwin F. Wagner

30.09.93  DAN FINLEY (Harvard Medical School, Boston)
  "Targeting proteins for turn-over via the ubiquitin pathway"
  Host: Kim Nasmyth
OCTOBER

04.10.93
THOMAS SHENK (Princeton Univ.)
"YY1: a transcriptional initiator and repressor whose activity is altered by the adenovirus E1A protein"
Host: Matt Cotten

05.10.93
GARY RUVKUN (Harvard Medical School)
"Generation of cell lineage asymmetry"
Host: Erwin F. Wagner

07.10.93
MICHAEL HALL (Biocenter, Basel)
"Phosphatidylinositol 3-kinase homologues TOR1 and TOR2 are required for cell cycle activation in yeast"
Host: Lisa Ballou

11.10.93
SABINE OBERT (SUNY at Stony Brook)
"Interaction of the adenovirus E4-6/7 protein with the cellular transcription factor E3F"
Host: Meinrad Busslinger

14.10.93
PAUL S. BURGOYNE (MRC, London)
"Sex chromosome effects on mammalian germ cell development"
Host: Denise Barlow

21.10.93
ANTHONY HYMAN (EMBL, Heidelberg)
"The role of the kinetochore in chromosome movement at mitosis"
Host: Kim Nasmyth

29.10.93
SOHALIA RASTAN (MRC, Middlesex)
"The mouse Xist gene: A candidate for the X inactivation center?"
Host: Denise Barlow

NOVEMBER

04.11.93
MICHAEL J. HAYMAN (SUNY at Stony Brook)
"Studies on the regulation of normal and abnormal proliferation and in avian hematopoietic cells"
Host: Hartmut Beug

09.11.93
MANFRED GOSSEN (ZMBH, Heidelberg)
"Tetracyclin controlled gene expression in higher eukaryotes"
Host: Hartmut Beug

18.11.93
ANDREW POMIANKOWSKI (Univ. College, London)
"Genomic conflicts underlying Haldane's rule and speciation"
Host: Denise Barlow

19.11.93
GERARD EVANS (ICRF, London)
"C-myc oncogene and tumor suppressor gene"
Host: Kim Nasmyth

25.11.93
CHARLES WEISSMANN (Univ. Zurich)
"Molecular biology of prion diseases"
Host: Matt Cotten

30.11.93
JACEK SKOWRONSKI (CSH; New York)
"Non-essential HIV-1 genes: HIV-1 nef function in vitro and in transgenic mice in vivo"
Host: Erwin F. Wagner

DECEMBER

01.12.93
DIETER C. GRUENERT (UCSF, San Francisco)
"Homologous replacement of specific sequences in somatic cells"
Host: Matt Cotten

07.12.93
PAUL MARTIN (Univ. College, London)
"Mechanisms of wound healing in embryos"
Host: Erwin F. Wagner

13.12.93
VOLKER SCHIRRMACHER (DKFZ, Heidelberg)
"Immunotherapy of metastasis: experimental and clinical studies"
Host: Matt Cotten

15.12.93
Carmen Birchmeier (Max-Delbrück-Lab., Cologne)
"Tyrosine kinase receptors in the control of epithelial cell growth, differentiation & morphogenesis"
Host: Erwin F. Wagner

16.12.93
AVRON MITCHEISON (DRFZ, Berlin)
"Down-regulation of the immune response"
Host: Kim Nasmyth

CASE 14/017
*Techniques for the production of tumor vaccines*
Inventors: K. Zatloukal, M. Cotten, E. Wagner, W. Schmidt, M.L. Birnstiel

CASE 14/018
*Virus for the transport of foreign DNA into higher eucaryotic cells*
Inventors: E. Wagner, M. Cotten

CASE 14/019
*Techniques for the production of tumor vaccines*
Inventors: E. Wagner, K. Zatloukal, M. Cotten, W. Schmidt, M.L. Birnstiel

CASE 14/020
*Endosomolytically active particles*
Inventors: E. Wagner, M. Cotten, S. Chiocca, G. Schaffner

I.M.P. Publications 1993


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organized by M.L. BirnstIEL and J.B. Gurdon
and session chairpersons
There will be 5 plenary lectures and
15 symposia.
Invited speakers include:
Anderson D (PasadenA), Box M (Cambridge,UK), Barlow D.P (Vienna),
Beddington R (Edinburgh), Boc'h H (Vienna), Bird A (Edinburgh), Brooks J.
(London), Buckingham M (Paris), Campos-Ortega J.A (Köln), Capechi M.R
(Salt Lake City), Cedar H (Jerusalem), Chambers P (Stellenbosch), Cleere T (Berkeley),
Cohen J (Denver), Cohen S (Houston), Davidson E (Pasadena), Dobilison D.
(Houdenberg), Edelman G M (New York), Eliav A (New York), Eichholz G.
(Houston), Elion J (Eugene), Erway T (London), Fraser S (Pasadena), Gelving
W.J. (Basel), Geshen A (Rheims), Goodenough D A (Boston), Grainger R, 
(Chicago), Grav P (Potsdam), Grash P (Göttingen), Gorden J (Cambridge, UK),
Haken E (Zürich), Hao C (Oxford), Hansbnam D (San Francisco), Harford R.
(Berkley), Haskle N (Edinburgh), Hemmern B.S (Tolandng), Hodgson J (Cambridge).
UK), Himmel M.F (Mediterr.), Horvitz R (Cambridge, USA), Hunt T (South Mimms),
Hyerce R (Cambridge,USA), Ingram P (Oxford), Iudice N (Toronto), Jack T.
(Cambridge,USA), Javinsch R (Cambridge,USA), Jan Y (San Francisco), Jack H.
(Göttingen), Jasse T M (New York), Johnson E J (St. Louis), Key P (Cambridge,UK),
Kiemer R (Freiburg), Keske J (Madison), Kreisel C (Eugene), Krüger C.
(San Diego), Krichner M (San Francisco), Korsmeyer S (St. Louis), Krompeich H.
(London), Koone A (Gron), Lassar A (Boston), Lawrence P (Cambridge,UK),
Le Douarin N (Nantes-Sur-Marne), Leifmann R (Cambridge,USA), Lemischka.
(New Jersey), Lovell-Badge R (London), Lomberd 4 (London), Maks M.
(Toronto), Martinet-Arias A (Cambridge,UK), Mc Ginnis W (New Haven),
Mcmahon A.P (Nottingham), Medcalf F (Basel), Meyrowitsz E M (Pasadena),
Meehny K (Vienna), Neufeld R (Zürich), Nusslein-Volhard Ch (Tolandng),
O'Connor G (Houston), Palmer N (Baltimore), Perel M (San Francisco), Pospisil J.
(Lu Jia), Platt M (London), Read D.W (St. Louis), Rubas G.M (Berkley),
Robroman J (Cambridge,USA), Scwartz L (Amsterdam), Simon M (Stanford),
Smith J (London), Sonigo P (Houston), Stanley R (New York), Staller H.
(Cambridge,USA), Stem C (Oxford), Suter A (Cambridge,UK),
Takahshl K (Kyoto), Thery J-P (Paris), Tinglef S (New York),
Tomlinson A (New York), Vincer J-P (San Francisco),
Wagner E (Vienna), Wall B (Pasadena), Wolffm M (Ithaca),
Wolpert L (London), Zeller R (Heidelberg)

Ample space and time will be allocated
for poster sessions.
For further information write before
Febru. 15th to:
INTERCONVENTION
KONGRESSORGANISATION
Mr. Oliver Butz

Austria Center Vienna
Am Huberstrasse 6 A-1450 Vienna
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