Instituto Juan March
de Estudios e Investigaciones

31 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Roles of Growth and Cell Survival Factors in Vertebrate Development

Organized by

M. C. Raff and F. de Pablo

Y.- A. Barde
H. Beug
Y. Courtois
A. Efstratiadis
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T. M. Jessell
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E. Mitrani - Growth Factors during Axis Formation in the Chick Embryo.

G. Martin - The Function of FGFs in the Developing Limb and Mammalian Hair Growth Cycle.

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INTRODUCTION

M. C. Raff

Recent events have transformed developmental biology beyond recognition. Ten years ago it would have been hard to find a developmental biologist who felt confident that, in his or her lifetime, we would understand the basic principles underlying the development of a fertilised egg into a mature organism. Today many feel such confidence. Both technical and conceptual advances have contributed to this new optimism. Recombinant DNA technologies have given us the tools to identify and manipulate virtually any macromolecule in an organism. The discovery that the fundamental mechanisms of development have been remarkably conserved in evolution has given us confidence that what we learn from genetic analyses of yeasts, flies and worms is likely to be directly applicable to vertebrates.

Among the many recent advances in developmental biology, few have been more spectacular than the explosive progress in identifying the signalling molecules that control animal development by regulating cell survival, proliferation, commitment, differentiation, adhesion and migration. Some of this progress is summarized in the following abstracts of the talks and posters that were presented at the recent Juan March Workshop on this subject held in Madrid.

I. Induction and Patterning in Early Development

RESPONSE TO GROWTH FACTOR SIGNALLING IN XENOPUS DEVELOPMENT

J. B. Gurdon, P. Lemaire, K. Kato, M. Weston.
Wellcome CRC Institute, Tennis Court Road, Cambridge CB2 1QR, England.

All experiments reported concern the earliest induction in Xenopus development, namely that which leads to the formation of the mesoderm and subsequently muscle, notochord, etc. There are four major steps in the derivation of muscle and notochord from the egg.

Contrary to general belief, not all aspects of Xenopus mesoderm formation depend on the mesoderm-forming induction. Previous work, involving ligation of fertilized single-cell eggs indicated the ability of cells to form mesoderm in the absence of an induction between vegetal and animal cells. Recent work with P. Lemaire has shown that the genes Xgoosecoid and Xwnt8, both mesoderm-specific genes, can be activated to nearly their full extent in the complete absence of cell interactions. Conversely, the gene Xbrachyury, another early mesoderm-specific gene, seems to be entirely dependent upon cell interactions.

The traditional mesoderm-forming or Nieuwkoop induction brings about a major increase in the abundance of transcripts of such genes as MyoD.

The mesoderm-forming induction is complete by the early gastrula stage, and yet the mesoderm cells formed about this time are not able to proceed to fully and stably activate muscle-specific genes, such as XMyoD or X12101 in the absence of further cell interactions. These other interactions between like cells are named a community effect. We have recently shown that the community effect is required by notochord cells shortly before the time when the same interaction is required for muscle differentiation. The community effect is believed to be important in helping to create uniformly differentiated groups of cells sharply demarcated from their neighbours which differentiate in other directions.

Recently with K. Kato, we have shown that there is a strong inhibitory influence from the ventral ectoderm during gastrulation which seems to counteract the community effect, and to demarcate the ventral limit of muscle differentiation from the mesoderm.

CADHERINS IN XENOPUS EMBRYOGENESIS

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I shall consider briefly some major features of the cadherin superfamily and provide examples of the potential role of some of its members in morphogenesis. In order to study the role of cadherins in the establishment of the body plan in xenopus embryos, we have chosen to perturb their function by virtue of over-expressing the cytoplasmic domains of several cadherins expressed at the blastula, gastrula and neurula stages. mRNAs coding for truncated N-, E-, XB- and EP- cadherins, lacking the extracellular domain, were injected in each blastomere at the 32-cell stage. The descendant of the injected blastomeres were followed throughout the gastrula stage. Morphological defects were obtained in tissues derived from the injected blastomeres or in tissues interacting with cells expressing the truncated cadherins. Morphological defects were much more severe following injection of truncated E-, XB- or EP- cadherin than those obtained with deleted N-cadherin constructs. In addition, the truncated E-cadherin often induced the formation of hyperplastic tissues. Noticeably, when the truncated cadherin mRNA was injected into blastomeres that contribute to neural induction, duplication of axis was observed.

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Growth factors during axis formation in the chick embryo.

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We have attempted to clone TGF-ß-related factors during early chick development. We have cloned TGF-ß2, a new gene Vgc1 and activin ßB from chick embryonic cDNAs. Vgc1 is the homologue of the mammalian gene BMP-6 and is expressed both in the epiblast and the hypoblast of the chick blastula. We have found that activin can induce the formation of axial structures including a full length notochord, segmented somites and a neural tube in isolated epiblasts from chick blastulae. We show that activin-ßB is transcribed, in the chick, precisely when axial mesoderm is being induced. We also show that activin can change the fate of competent cells and bring about the formation of an ectopic embryonic axis from a different subset of cells. Activin inhibits proliferation of early chick embryonic cells and probably acts by inducing differentiation of early embryonic cells into mesoderm. Furthermore we show data which suggest that during normal development only one axis is obtained because of a carefully controlled inhibitory process. We could not detect TGF-ß2 RNA during early development but, surprisingly we detected by RNAse protection and by directional cDNA an antisense RNA complementary to TGF-ß2. The effect of similar factors in controlling proliferation in adult epidermis will also be discussed.

Gail Martin

The function of FGFs in the developing limb and mammalian hair growth cycle

We have been interested in determining the functions of different members of the FGF family in the developing embryo. Initially, we carried out RNA *in situ* hybrdization studies of the expression patterns of several FGF family members in the mouse embryo (Hébert et al., 1991; Niswander and Martin, 1992; Han and Martin, 1993). Based on the observation that *Fgf4* is expressed in the apical ectodermal ridge of the limb bud, which is known to be the source of signals required for limb outgrowth, we hypothesized that the FGF4 protein plays a key role in limb development. To explore this possibility we initially carried out experiments on cultures of intact mouse limbs from which the ridge had been removed, and demonstrated that FGF proteins can stimulate limb mesenchyme proliferation (Niswander and Martin, 1993a). We have also demonstrated that FGFs induce and maintain the expression in limb mesenchyme of *Evx-1*, a homeobox gene related to the *Drosophila even-skipped* gene (Niswander and Martin, 1993b). Subsequent studies on chick embryos *in ovo* have shown that recombinant FGF-4 can substitute for the ridge, and perform all the functions of the ridge required for virtually complete outgrowth and patterning of the limb (Niswander et al., 1993). In particular, studies in which FGF-4 was applied to different regions of the limb bud have provided evidence that it performs two functions essential for limb outgrowth and patterning: it stimulates proliferation of cells in the distal mesenchyme and maintains a signal from the posterior to the distal mesenchyme that appears to be required for elaboration of skeletal elements in the normal proximo-distal sequence. Based on our data we suggest a simple mechanism whereby FGFs produced in the ridge link growth and pattern formation during limb development. Studies currently in progress are aimed at understanding how *Fgf-4* expression in the ridge is regulated.

Our RNA *in situ* hybridization data also suggested that the *Fgf5* gene might play a role in the developing embryo. To explore its function we used the gene targeting approach in embryonic stem cells to produce mice homozygous for a mutant allele of *Fgf5*. To our surprise the mutant homozygotes were found to be healthy and fertile, and appear phenotypically normal until approximately three weeks after birth, when their hair becomes noticeably longer than that of their heterozygous and wild-type littermates. This long-hair phenotype is apparently identical to that of mice homozygous for the spontaneous mutation *angora*. Subsequent analysis has revealed that the *angora* mutation in mice is due to a deletion that includes *Fgf5* coding sequences, and probably the promoter of the gene as well. Our data indicate that *angora* is a mutant (null) allele of *Fgf5*, and that in the hair follicle a normal function of FGF-5 is to inhibit hair growth. In the absence of FGF5 a key step in progression through the hair growth cycle, the transition from the

phase of active hair growth to that of follicle regression is substantially delayed. These results provide the first identification of a molecule that regulates one step in the progression of the hair follicle through the hair growth cycle and provide a starting point for studies of the molecular basis of hair growth control.

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II. Insulin, FGFs, IGFs and TGF in Development

FGF1, a survival factor in retinal development and physiology

Yves Courtois, Flore Renaud, Sophie Desset, Jean-Marc Philippe, Lisa Oliver, Edith Jacquemin, Jean-Claude Jeanny, Maryvonne Laurent. INSERM U.118, Affiliée CNRS, Association Claude-Bernard, 29, rue Wilhem, 75016 Paris, France

During retinal development FGF1 expression follows the pattern of cell differentiation [1, 2]. Its expression (mRNA and protein) starts to increase sequentially first in ganglion cells, then in intermediate neurones and in photoreceptors. It then remains highly expressed. The regulators of this phenomenon are not yet understood. Since the aFGF gene organisation is complex, with tissue specific untranslated exons [3], the promotors are still not fully characterized.

Most of the experiments to unravel the role of FGFs have dealt with the paracrine mechanism by adding exogenous FGFs to various biological models. For instance, FGF1 and FGF2 directly induce photoreceptors differentiation in new born rat retinal culture [4]. But very little is known about the role of endogenous expression which may act through an autocrine or intracrine pathway.

To determine the role of endogenous FGF1 in non mitotic conditions, we analysed the regulation of its expression in two cell systems. **1.** In bovine lens cells (BEL) which are held out of the cell cycle. **2.** In PC12 cells in which FGF1 is constitutively over expressed by permanent transfection.

1. In BEL cells arrested either by contact inhibition or by serum starvation FGF1 (transcripts and proteins) is over expressed compared to exponentially growing cells implying that endogenous aFGF has no mitogenic role under these conditions. Moreover the addition of oligo antisense primers to these culture leads to cell death. These results suggest a direct survival role for endogenous FGF1 [5].

2. This was confirmed recently in PC12 cells. In these cells FGF1 is also expressed endogenously and its amount increases with their differentiation in neurones. Exogenous FGFs auto induce FGF1 synthesis. To discriminate between the respective ability of endo FGF1 versus exo FGF1 we have transfected PC12 permanently with constitutive or inducible FGF1 constructions. The presence of neurite extensions in transfected cells depended on the level of endo FGF1 produced by these cells. A moderate increase in endo FGF1 is enough to allow for the survival of the differentiated cells for several weeks in serum free culture medium.

All these data provide evidence that endo FGF1 acts as a neurotrophic factor by a mechanism which may be independant of the canonical tyrosine kinase receptor activation pathway.

It will be of interest to demonstrate that a similar role for FGF1 is involved in the development and physiology of the retina.

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NON-PANCREATIC INSULIN AND IGF-I AS PROLIFERATION/ DIFFERENTIATION FACTORS IN EARLY DEVELOPMENT

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Members of the insulin family of polypeptides include insulin, its precursor proinsulin, and the insulin-like growth factors I (IGF-I) and II (IGF-II). Their signaling receptors, the insulin receptor and the IGF-I receptor, belong to the class of protein tyrosine kinase receptors that autophosphorylate upon activation, and phosphorylate endogenous substrates such as IRS1.

Despite the similar pleiotropic effects of insulin and IGF-I in cultured cells, affecting proliferation, differentiation, cell survival and metabolic regulation, the prevailing view until recently considered insulin almost exclusively a pancreatic hormone engaged in the control of metabolism, and IGF-I a tissue growth factor with a postnatal endocrine role, promoting longitudinal bone growth. Several lines of research now lead to a redefinition and broadening of the possible roles of insulin and IGF-I during development, acting as both endocrine and paracrine factors.

Maternal insulin and IGF-I are stored in the egg of chicken and *Xenopus* (1). Insulin receptors and/or IGF-I receptors are expressed and active in very early chicken (2,3), *Xenopus* (4) and *Drosophila* embryos (1). We demonstrated that expression of insulin mRNA, evidenced by Northern analysis, anteceded the differentiation of the pancreas in chicken embryos (E2), and that insulin antibodies or insulin receptor antibodies applied at that stage resulted in embryonic growth retardation or arrested development (5). Confirmation of prepancreatic insulin gene expression in mammals as well, has been reported (6).

In more recent studies, we have used a highly sensitive modification of RT-PCR to study the levels of insulin and IGF-I gene expression from the unincubated blastoderm stage until late neurulation (E0-E2). Insulin mRNA is expressed at all stages, with maximum levels during gastrulation. *In situ* hybridization analysis of embryos *in toto,* showed a wide distribution of insulin mRNA in a pattern similar to insulin receptor mRNA and radiolabelled insulin

binding. IGF-I mRNA expression appeared later than insulin mRNA, in the embryo and predominated in the head portion at E2-E3 (7).

With a novel culture system for chick embryos during neurulation, we compared regulation by glucose of prepancreatic and pancreatic insulin gene expression . While pancreatic insulin mRNA increased two-fold in the presence of 17 mM glucose, the prepancreatic embryo insulin mRNA was unchanged, suggesting differential regulation of this early insulin transcripts. Exogenous insulin added to the culture increased protein and DNA synthesis in E1.5 embryos, supporting the relevance of insulin signaling at this stage. Prepancreatic insulin, thus, acting in paracrine fashion, may be the member of the family with the earliest role in embryonic development. Ongoing studies using antisense oligonucleotides and cultured embryos may clarify whether this role is to support cell survival and proliferation of many of the early embryo cells or whether some specific cell types, for instance neural precursors, are more insulin dependent.

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Role of Insulin-like growth factor binding proteins in fetal development.

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The insulin-like growth factors, IGF-I and IGF-II, are cytokines involved in cell-to-cell signalling (1-3). They act principally through IGF-I receptors, tyrosine kinase receptors that are homologues of insulin receptors, to stimulate cell growth and survival, as well as other functions. The IGFs and IGF-I receptors are expressed in many tissues in the embryo and postnatally. As discussed by Dr. Efstratiadis, inactivation of the mouse genes for IGF-II, IGF-I, and the IGF-I receptor, separately and in combination, have established that all three genes are required for the birth of fetuses of normal body weight.

A third component of the IGF system, the IGF-binding proteins (IGFBPs), also regulates IGF action (2,3). The IGFBPs are a family of six related proteins that bind IGF-I and IGF-II with high affinity, but do not bind insulin. They are products of distinct genes that are unrelated to the genes for the IGFs and IGF receptors. The IGFs in plasma, tissue fluids and cell culture media always occur bound to one of the IGFBPs. IGF : IGFBP complexes typically are inactive, because they cannot bind to the IGF-I receptor. In some circumstances, however, the IGFBPs may potentiate the action of IGFs on target cells (2,3). Because they are regulated independently and have distinctive properties, the IGFBPs provide a flexible and versatile system for the regulation of IGF action.

All six IGFBPs are expressed early in embryogenesis (4-6). They frequently have exquisitely specific expression which in some cases is distinct from that of other IGFBPs, suggesting potential biological function, and in other cases is overlapping. For example, in mid-gestation rat embryos, IGFBP-1 is expressed only in liver, IGFBP-4 is expressed strongly in liver, whereas IGFBP-5 is not expressed in liver (5). Both IGFBP-5 and IGFBP-3 mRNAs are expressed in muscle (5). Localized expression of IGFBP-2 in the floor plate and infundibulum of mid-gestation embryos (4) and in the apical ectodermal ridge (7) suggested a possible role in central nervous system and limb development. However, homozygous IGFBP-2 knockout mice exhibit normal growth and viability, and have no morphological defect (8). Presumably other IGFBPs compensate for the loss of IGFBP-2 function.

Transcription of the IGFBP-1 and IGFBP-2 genes in rat liver is developmentally and metabolically regulated (9). To understand this regulation, we have begun to study the rat IGFBP-1 and IGFBP-2 promoters. The proximal rat IGFBP-1 promoter contains adjacent *cis*-elements that determine induction by glucocorticoids and inhibition by insulin (10). Transcription of the rat IGFBP-2 promoter originates at a discrete transcription initiation site despite the absence of TATA and initiator sequences (11). The GC-rich proximal promoter region contains 3 clustered Sp1 sites which function synergistically and are required for basal promoter activity.

In addition to synthesis, post-translational modifications (such as glycosylation, phosphorylation, and proteolysis) of the IGFBPs and interaction with extracellular matrix and the cell surface also may affect IGFBP activity. It has been proposed that soluble IGFBPs which bind IGFs with high affinity inhibit IGF action by preventing the IGFs from binding to their receptors, whereas IGFBPs that localize to the cell surface or extracellular matrix potentiate IGF action (2,3). The affinity of the IGFBP for IGF also

must be decreased for potentiation to occur. This might result from association with the cell surface or matrix, proteolysis, or dephosphorylation. We have begun to test this hypothesis using recombinant human IGFBP-6 (12). Recombinant IGFBP-6 synthesized in COS-7 monkey kidney cells, like native IGFBP-6, is an O-linked glycoprotein that binds IGF-II with marked preferential affinity over IGF-I. The purified recombinant protein inhibited IGF-II-induced differentiation of L6A1 rat myoblasts. The added IGFBP-6 remained in the culture medium and did not associate with cells or matrix. These results support the hypothesis that soluble, intact IGFBP-6 inhibits IGF-II action. Structural modifications will be introduced in IGFBP-6 to promote its localization to pericellular components, in the hope of converting it from an inhibitor to a potentiator of IGF action.

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POSTNATAL PHENOTYPE OF MOUSE *Igf1* NULL MUTANTS

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The *in vivo* growth-promoting role of insulin-like growth factors (IGFs) has been demonstrated from the results of targeted mutagenesis of the mouse genes that encode IGF-I, IGF-II and IGF1R (type-1 IGF receptor). Comparisons of the phenotypic manifestations of these dwarfing null mutations, alone or in combination, in terms of survival, developmental delays in particular tissues, severity of growth deficiency, and embryonic growth kinetics, defined the onset of mutational effects and indicated that IGF1R serves *in vivo* the signaling of both IGF-I and IGF-II, while IGF-II recognizes an additional, unknown receptor. Studies with embryonic fibroblasts from the mutants lacking IGF1R showed that the cell cycle is 2.5-fold longer than normal, indicating that this signaling system influences the most important growth determinant: the rate of cellular divisions that increase total cell number.

In contrast to IGF-II, which has a predominantly embryonic role in rodents, IGF-I functions both during embryonic and postnatal development. Moreover, the *Igf2* null mutants are viable and fertile, and grow with a normal rate postnatally, while the survival of *Igf1* null mutants is variable, depending on genetic background, and their postnatal growth rate is reduced. Thus, they exhibit a birthweight that is 60% of normal, but their size becomes only 30% that of their wild-type littermates at 4 weeks of age. Analysis of long bone development has shown a decrease in the number of osteoblasts and stromal cells in the *Igf1* null mutants and a delay in the closure of the growth plate. However, the most dramatic impact of the *Igf1* null mutation is on reproductive functions. Thus, both sexes lack libido and are infertile. The males have testes allometric to their reduced body size and sustain spermatogenesis, although the normal sperm, which can fertilize wild-type eggs *in vitro*, is produced at low numbers (10% of normal). Moreover, their accessory ducts and glands are vestigial in size, while their serum testosterone concentrations are low (by electron microscopy, Leydig cells do not appear to differentiate beyong days 10-14 of postnatal development). The female mutants posses an infantile uterus (11% of normal weight) and do not cycle through estrus. The mutant ovaries contain follicles at several early stages of development, but no preovulatory Graffian follicles or corpora lutea, which indicates that these animals fail to ovulate (attempts for superovulation by administration of gonadotrophins were unsuccessful). These results provide the first direct evidence that the IGF system is involved in reproductive functions. It is likely that the common primary defect in both sexes of *Igf1* null mutants is an impairment of mechanisms participating in gonadal steroidogenesis.

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TGF-ß FORMATION; MECHANISMS AND CONSEQUENCES

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TGF-ß, as normally found in tissues and media conditioned by cells, is composed of a high molecular weight complex in which the active growth factor remains noncovalently bound to its propeptide. This complex, sometimes known as small latent complex, is inactive and will not react with TGF-ß high affinity receptors. In addition a higher weight molecular complex exists that consists of the small latent complex plus a second gene product, the latent TGF-ß binding protein (LTBP), disulfide bonded to the propeptide. The large latent complex also does not interact with high affinity TGF-ß receptors. We have found that conversion of latent TGF-ß to TGF-ß requires a cell surface assemblage of proteases and binding factors. The required proteases are plasminogen activator and plasmin. The components of the larger latent complex that interact with the cell and/or matrix appear to be the LTBP and mannose-6-phosphate residues on the propeptide. In addition, the enzyme transglutaminase is necessary for activation as inhibitors of tissue transglutaminase block activation. These requirements govern TGF-ß formation in cocultures of vascular cells as well as homotypic cultures of either activated peritoneal macrophages or endothelial cells treated with agents such as retinoids. In the case of cocultures of endothelial and smooth muscle cells, the endothelial cells supply the proteolytic component, plasminogen activator, as well as the transglutaminase, whereas the smooth muscle cells provide binding sites for the latent TGF-ß.

Because the amounts of TGF-ß formed in these experiments are relatively low (20-50 pg/ml), we have developed a bioassay for TGF-ß that employs a chimeric cDNA comprising a region of the PAI-1 promoter fused to the luciferase gene. This construct was transfected into mink lung epithelial cells and permanent transfectants isolated. These transfectants, when exposed to TGF-ß, respond with increased expression of luciferase that can be quantitated automatically in a luminometer using 96 well dishes. This assay yields linear responses within a range of 5-200 picrograms of TGF-ß per ml. The response is reatively specific as basic fibroblast growth factor PDGF, EGF, and LPS have no effect on the assay.

We have recently begun to explore the regions of the LTBP required for activation. We have made expression vectors that include regions of the LTBP derived from the amino-terminus, the central region, and the carboxy-terminus of the protein fused to GST. When these peptides were tested in activation assays, the peptide representing the carboxy-terminal region blocked activation, whereas the peptides representing the amino-terminal or the central regions of the molecule had no effect. Antibodies prepared against each of these peptides were tested in activation assays for their ability to inhibit production of TGF-ß. The antibody against the caroxyl-terminal region blocked activation, whereas antibodies against the amino-terminal and central regions of LTBP were inactive. The antibodies and peptides to LTBP inhibitied TGF-ß generation in both cocultures of endothelial and smooth muscle cells and activated mouse peritoneal macrophages.

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III. Hemopoiesis

Self renewing, erythroid progenitors develop from CFU-E, requiring at least two receptor tyrosine kinase ligands and one nuclear hormone receptor ligand

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Recently, we described that normal hematopoietic progenitors committed to the erythroid lineage were capable of sustained self renewal under specific conditions, i.e. by the combined action of TGFα (a ligand for the avian homolog of the epidermal growth factor receptor/c-erbB protooncogene) and estradiol. These cells, (referred to as SCF/TGFα progenitors) expressed the the c-*kit* proto-oncogene, the estrogen receptor and TGFαR/c-*erbB* and were capable of sustained self renewal in presence of TGFα plus estradiol until the end of their normal *in vitro* lifespan. We also demonstrated that erythroid progenitors indistinguishable from normal CFU-E by all properties studied (referred to as SCF progenitors) could be grown out from bone marrow by avian stem cell factor (SCF). These SCF progenitors lacked the expression of TGFαR/c-*erbB* and showed only transient self renewal for 7-10 days in presence of SCF. When switched to differentiation factors (Epo plus insulin), both types of progenitors differentiated into erythrocytes with indistinguishable kinetics. This indicated that SCF/TGFα progenitors are not the precursors of SCF progenitors, as initially suggested by the fact, that SCF/TGFα progenitors are relatively rare (1 in 15,000 normal bone marrow cells), while the SCF progenitors are much more frequent (1 in 3-500).

Our results, however, left open the question about the developmental origin of the self renewing SCF/TGFα progenitors. i.e whether they represented a separate, rare cell type preexisting in bone marrow or originate from normal CFU-E. Results will be presented clearly favouring the second alternative. If normal SCF progenitors are cultivated in a combination of SCF, TGFα, estradiol and unknown factors from normal or anemic chicken serum, a large proportion of these cells undergo neither differentiation nor apoptosis, but continue to proliferate and start to express increasing levels of TGFαR/c-*erbB* in a strictly time-dependent process, yielding self renewing SCF/TGFα progenitors after 10-14 days. At this time, TGFαR/c-*erbB* expression in the cells is apparently high enough to allow proliferation in presence of TGFα and estradiol, but absence of SCF. By using suitably stripped chicken sera, we could show that no SCF/TGFα progenitors are generated if one of the three factors (SCF, TGFα or estradiol) is absent. On the other hand, the generation of the self renewing progenitors in presence of SCF, TGFα and estradiol was partially inhibited, but not abolished, if the unidentified activity in chicken serum was absent.

EARLY MATURATION EVENTS IN THE DEVELOPMENT OF HUMAN PRETHYMIC AND INTRATHYMIC HEMATOPOIETIC PRECURSORS.

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Hematopoietic stem cells home to the fetal liver at 5-6 wk of human embryogenesis. The liver then becomes, until week 24, the major site of hematopoiesis, supporting the proliferation of progenitor cells for all hematolymphoid lineages. Differentiation of these progenitor cells along a particular hematopoietic lineage is further dependent upon epigenetic influences provided by specific cellular microenvironments. The development of immunocompetent T lymphocytes occurs primarily in the embryonic thymus, where both cellular interactions between developing thymocytes and thymic stromal components, as well as the coordinated production of multiple growth factors, are critically involved in the T-cell developmental program.

We have addressed the study of the early maturation events which control the differentiation of hematopoietic progenitors (either before or after their homing to the thymus), along the T lymphoid lineage. Hematopoietic precursors present in the liver in early human fetal life were shown to express different adhesion molecules (VLA-4, LFA-3) and activation antigens (4F2, transferrin receptor), before they acquired specific markers for the T-cell, B-cell or myeloid lineages. Molecular studies showed that transcription of the interleukin-2 receptor β chain (IL-2Rβ) gene and expression of IL-2Rβ proteins also ocurred in the embryonic liver at this early ontogenic stage. In contrast, no expression of IL-2Rα or IL-2 transcripts was detected, whereas transcription of the IL-4 gene was observed in a small fetal liver cell subset. Putative T-cell precursors were identified among the hematopoietic fetal liver cells by the expression of genes encoding the γ, δ, ε, and ζ invariant chains of the CD3-T cell receptor (TCR) complex. However, the polymorphic α and β TCR genes were still in germ line configuration, and no mature transcripts were detected (1).

Functional *in vitro* assays further demonstrated that early fetal liver hematopoietic precursors were capable of proliferating in response to T-cell growth factors, including IL-4 and IL-2. Proliferation was shown to be accompained by the acquisition of T-cell- as well as myeloid-specific markers, although differentiation into mature $CD3^+$ T lymphocytes was not detected. In fact, IL-4-induced proliferation paralleled the appearance *in vitro* of $CD45^+CD7^-CD4^{dull}$ cells expressing the CD14 myeloid antigen, as well as of $CD34^+$ primitive hematopoietic progenitors, while differentiation into $CD45^+CD7^+CD8^+CD3^-$ cells was observed when using IL-2. Interestingly, both $CD34^+$ and $CD45^+CD7^+CD4^-$

$CD8^-$ hematopoietic progenitors were also obtained when fetal liver cells were cocultured with thymic epithelial cell (TEC) monolayers in the absence of exogenous cytokine supplementation (1). The observation that TEC lines used in our cultures, as well as TEC clones derived from them, expressed functional transcripts specific for different growth factors including IL-1, IL-5, IL-6, IL-7, IL-8 and TGFβ1 (2), led us to analyze whether cytokines produced by the thymic epithelium could be responsible for the survival of $CD34^+$ and $CD45^+CD7^+CD4^-CD8^-$ hematopoietic precursors *in vitro* . To this end, $CD34^+$ early precursors were isolated from the neonatal thymus and cultured in the presence of different cytokines. These studies revealed that proliferation of both $CD34^+$ and $CD34^-$ $CD7^+$ thymic precursors was supported by IL-7. More interestingly, IL-7 was also able to induce the differentiation of thymic precursors into immature $CD1^+$IL-2Rα$^+CD3^-$ pre-T cells, and later on into $CD1^+CD4^+CD8^+CD3^-$ double positive thymocytes. Differentiation of intrathymic precursors along the T-cell lineage was accompained by the simultaneous development of cells expressing myeloid and NK markers. Further studies showed that, as observed for IL-7, TNF-α behaves as a survival factor for thymic-derived myeloid cells, while GM-CSF was able to induce the proliferation of this myeloid progeny. Proliferation of NK cells was achieved when either TNF-α or IL-2 was provided to the cultures. Taken together, these results support previous data suggesting that, in addition to T-cell precursors, progenitors of other hematopoietic lineages are included within the subset of early thymocytes.

Finally, to address whether additional costimulatory signals delivered *in vivo* within the thymic microenvironment were required to efficently drive terminal differentiation of primitive hematopoietic progenitors along the T-cell lineage, TEC lines derived from neonatal or fetal thymic samples were cocultured with either prethymic or intrathymic precursors in the presence of IL-2. These studies provided final proof that early fetal liver hematopoietic cells, as well as $CD34^+$ intrathymic progenitors, include very primitive T-cell precursors which were able to differentiate *in vitro* into TCR α/β^+ mature T cells. Therefore, our results indicate that, after triggering of the T-cell-specific maturation program in primitive fetal liver hematopoietic progenitors, specific signals provided intrathymically by epithelial cells may fullfil the requirements to drive terminal differentiation of prethymically committed T-cell precursors.

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The Control of Hemopoiesis by Regulatory Factors

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The formation of blood cells is a developmental process that continues actively throughout life. The required formation of cells in eight major developmental lineages occurs in restricted specialized tissues where there is evidence that special stromal cells interact with adjacent hemopoietic cells to create a permissive microenvironment of particular importance for the formation of committed progenitor cells from self-renewing hemopoietic stem cells. In parallel with this local cell contact control, a group of specific hemopoietic regulators plays a dominant role in controlling mature cell production by committed progenitors.

More than twenty specific hemopoietic regulators have been identified and cloned and some, such as erythropoietin and G-CSF are in extensive clinical use to promote hemopoiesis.

The principal features of these regulators are well exemplified by the four colony stimulating factors - G-CSF, GM-CSF, M-CSF and Multi-CSF (IL-3) - that are major regulators of granulocyte and macrophage formation. All four are glycoproteins with the polypeptide exhibiting four α-helical bundles. The CSFs normally are produced in low concentrations by a wide range of cell types including stromal and endothelial cells, fibroblasts, macrophages and lymphocytes. Their half-life in the serum is 1-3 hours and levels of transcription and protein production can be rapidly amplified in response to inducing signals - most often of microbial origin.

The CSFs interact with specific membrane receptors and in their high-affinity form they exist as either homodimers (for M-CSF and G-CSF) or heterodimers (for GM-CSF and Multi-CSF) - the latter two receptors sharing a common β-subunit.

Signalling by CSFs is mandatory for cell division to occur in immature granulocytes and monocytes, the concentration (0.5-4.0 ng/ml) controlling transit of the cells from G1 to S and determining the total cell cycle time and thus the total number of progeny produced.

Each CSF also controls viability of granulocytes and macrophage cells by maintaining membrane transport integrity, differentiation commitment, maturation induction and the functional activity exhibited by the mature cells. These various functions require multiple signalling pathways to be activated by each occupied CSF receptor and these signals are initiated by differing regions of the cytoplasmic domain of the receptors.

The regulatory control system exhibits considerable functional overlap because individual cells coexpress receptors for more than one CSF. However the system is not a redundant one as gene inactivation studies document specific defects resulting from inactivation of each CSF gene. Indeed the action of the multiple CSFs achieves a more efficient cell production, subtle changes in the range of mature cells produced and selective localization of the cells produced.

As best exemplified by the homology between many of the hemopoietic growth factor receptors, the present complex of regulators has probably evolved from a common ancestral regulator, the present day complexity being designed to cope with the demands of multicellular organisms.

Recombinant CSFs produced in bacteria or yeast, on injection are able to stimulate granulocyte and/or monocyte formation in vivo and have their widest current clinical use in promoting hemopoietic regeneration in cancer patients following chemotherapy and/or marrow or peripheral blood stem cell transplantation.

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DISRUPTION OF MESODERM AND AXIS FORMATION IN FISH BY ECTOPIC EXPRESSION OF ACTIVIN-VARIANTS: THE ROLE OF MATERNAL ACTIVIN

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One of the basic questions of developmental biology is how a radially symmetrical egg is developing into an organism with all three body axes. Classical experiments with amphibian embryos have demonstrated that the mesoderm is playing a crucial role in this process. Induction of mesoderm and the factors involved are therefore believed to be the key players in the process of axis formation. I choose the Japanese medaka (Oryzias latipes) as system to study the role of activins in mesoderm induction. There is experimental evidence that the factors and the mechanism involved in embryonic induction in fish is quite similar to those utilized by in higher vertebrates. The transplantation of the trout archenteron roof induces the formation of a secondary body axis. The zebrafish embryonic shield acts as an organizer across species borders when transplanted to triturus.

I could demonstrate the presence of activin crossreactive protein in the nucleus of fully grown medaka oocytes and in the 1-cell embryo of medaka. I cloned the medaka and zebrafish activin ßa and ßb cDNAs by a PCR based approach and demonstrated activity of the expressed protein in the animal cap assay. Injection of synthetic RNA into the 2-cell embryo of medaka led to a high rate of multiple axis formations.

To directly address the question if activins are involved in mesoderm induction and axis formation *in vivo* there is an obvious need to directly interfere with the endogenous gene products and eliminate their activity. I created two dominant negative variants interacting with wild type (wt) activin. One of the variants (Pt) acts as an antagonist of mature activin protein. It suppresses activin induced induction of mesoderm in the animal cap assay as assayed by the induction of mix.1 and cardiac actin. The second variant (CS) depletes the pool of active activin, but only when coexpressed with wt activin.

RNA encoding these two variants were injected into the 2-cell embryo of medaka. Pt RNA is suppressing mesoderm and axis formation in a dose dependent manner. Expression of mesodermal markers as Brachyury protein and notochord is abolished by injection of 500 fg of Pt RNA whereas I do not find any sign of enhanced expression of N-CAM in those embryos. The injection into both cells of the embryo of CS RNA that acts dominant negatively only when coexpressed with wt activin did even in highest amounts (120 pg) not lead to any effect. The suppression of mesoderm induction as induced by the Pt variant can be rescued by titrating it out by the coinjection with CS-RNA.

This allowed to clearly demonstrate that activin is *in vivo* required for mesoderm induction and axis formation in fish. Furthermore it demonstrates that the maternally provided activin protein, not the zygotically transcribed activin is necessary for these processes *in vivo*.

CONTROL OF CELL BEHAVIOUR DURING VERTEBRATE DEVELOPMENT BY *Slug*, A ZINC FINGER GENE.

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Slug is a novel chick gene encoding a zinc finger protein of the *snail* family. Analysis of the pattern of expression by whole mount *in situ* hybridisation shows that it is expressed in neural crest and its derivatives and in early mesodermal cells emigrating from the primitive streak.

Because early development in birds closely resembles that in mammals, but can occur in a culture system where it can be observed continously, we have designed antisense oligonucleotides to interfere with *Slug* function.

Incubation of chick blastoderms with these oligonucleotides consistently causes inhibition of the epithelial-mesenchymal transition. This cell behavioural transition occurs at two principal sites within the emrging body plan; the formation of mesoderm during gastrulation and emigration of neural crest from the neural tube.

IV. Neural Development I

"GROWTH FACTORS IN INNER EAR DEVELOPMENT"

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The development of the vertebrate inner ear starts with induction of the otic placode from the presumptive ectoderm. The otic placode invaginates and develops into the otic vesicle, which then grows and differentiates into the adult inner ear, containing the highly specialized auditory and equilibrium sensory organs. Classical experiments showed that the induction of the otic vesicle requires of signals that originate from the mesoderm and the neural tube. The expression of the proto-oncogene *int-2* (**FGF3**) has been demonstrated in the neural tube, close to the otic presumptive area (Wilkinson, 1988). **Int-2** belongs to the fibroblast growth factor (FGF) family and encodes several products with mitogenic capacity. The suggestion that *int-2* may be part of inductive signals is supported by the pattern of distribution of the **int-2** onco-protein, and by experiments showing that the formation of the otic vesicle is inhibited by antisense oligonucleotides targeted against *int-2* (Represa et al., 1991).

While the otic vesicle is formed, specified neuroblasts migrate from the otic epithelium to form the cochleo-vestibular ganglion (CVG). The CVG contains all neurones connecting receptor hair-cells to the central nervous system. Both the otic vesicle and the CVG undergo an intense period of cell proliferation before cell differentiation is initiated, and several growth factors differentially regulate cell-division in the otic vesicle and the cochleo-vestibular ganglion. Among growth-factors, **IGF-I** is the most potent mitogen for the otic vesicle 'in vitro', strikingly mimicking normal proliferative growth at nanomolar concentrations. **IGF-I** factor and receptors can be demonstrated in the otic vesicle (León et al., 1994). Mitogenic effects of **IGF-I** appear to be coupled to the hydrolysis of a membrane glycosyl-phosphatidyl-inositol (GPI) and the generation of a soluble inositol-phospho-glycan (IPG), as well as to the expression of the nuclear proto-oncogene *c-fos*. **IGF-I** induce Fos expression, and *c-fos* antisense or anti-IPG antibodies result in growth arrest. **IGF-I** emerges as a good candidate to regulate cell-division during normal development of the inner ear.

Members of the NGF-family of neurotrophins (**NGF, BDN, NT-3**) exert distinct effects on developing cochlear neurones, including stimulation of cell-proliferation during early stages and survival and neuritogenesis during innervation periods (Represa et al, 1991; Avila et al., 1993). Early mitogenic effects appear to be coupled to the hydrolysis of membrane GPI. Differentiation effects, however, require high-affinity binding sites and are clearly discriminative for **BDNF** and **NT-3** against **NGF**. Biological effects overlap with the differential expression of low (p^{75}) and high-affinity receptors. The latter belong to the *trk* proto-

oncogene receptor family which expression is restricted to neurones within the cochlear ganglion and regulated throughout development. Experiments also show that otic-conditioned media are able to mimic some of the effects produced by neurotrophins, like stimulation of neurite outgrowth and other phenotypic characteristics in cultured cochlear neurones. Similar studies have been performed on vestibular neurones which are associated with the sense of equilibrium and postural reflexes, and that have a similar embryological origin. These and other results indicate that the **NGF**-family of neurotrophins are good candidates to regulate the development of auditory and vestibular neurones. Specificity of response is achieved by spatial and temporal restriction of factors and receptors and by different intracellular messenger mechanisms, resulting in regulation of cell-division or differentiation of receptor and neuronal networks.

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Control of Cell Differentiation in the Neural Tube

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During the development of the vertebrate nervous system distinct cell types are generated at defined positions along the dorso-ventral axis of the neural tube. Grafting experiments in chick embryos have shown that floor plate cells are induced at the ventral midline of the neural tube in response to local signals from the notochord. The notochord and also the floor plate can induce motor neurons and other ventral neuronal cell types but suppress the differentiation of dorsal cell types. Conversely, elimination of the notochord prevents floor plate and motor neuron differentiation and results in certain dorsal cell markers appearing at all dorso-ventral positions within the neural tube. These results indicated that signals from notochord and floor plate control the differentiation of distinct cell types in the ventral neural tube, although the differentiation of dorsal neural cells is not dependent on these signals. The molecular nature of these ventralizing signals will be discussed.

The acquisition of dorsal cell properties could represent a constitutive fate of neural plate cells. However, studies on dorsalin-1, a dorsally restricted member in the TGF-β gene family, has suggested that cell patterning in the dorsal neural tube is under more active regulation. Exposure of neural plate cells to dorsalin-1 promotes the differentiation of cells with neural crest-like properties and inhibits the induction of motor neurons by signals from the notochord and floor plate. The nature of signals involved in the specification of dorsal cell types will be discussed.

The Role of Cell Signaling in Development of the Vertebrate Embryo.

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The importance of cell to cell signaling in vertebrate development has been appreciated since the turn of the century, however only recently have we approached a molecular understanding of these processes. Our studies have focused on the role of three distinct classes of signals; Wnts, Hedgehogs (Hhs) and Noggin in the early mouse and chick embryo. Wnts and Hhs are thought to encode short-range signaling molecules whereas the signaling mechanism of Noggin is less well studied. We have chosen to study four aspects of embryonic development; gastrulation, neural induction and CNS patterning, limb patterning and organogenesis of the kidney. Using mutational analysis in mice we have identified several members of these signaling families which are essential for these events. For example, Wnt-3a appears to function in the establishment of a specific mesodermal population, the somitic mesoderm, in a concentration dependent fashion. In the brain, midbrain development is absolutely dependent on Wnt-1 signaling, and diencephalic development requires two redundant signals, Wnt-1 and Wnt-3a. Later in CNS development members of the Wnt and Hh family play distinct roles in the establishment of dorsal-ventral polarity. Patterning of the vertebrate limb along the dorsal-ventral and anterior-posterior axes also requires specific Wnt and Hh signals and it is likely that these signaling pathways interact at some level. Finally, expression and mutational studies of Wnt-signalling in the mouse kidney suggests that several inductive steps we lead to the development of the nephron are regulated by Wnt-signaling. I will review our studies that lead to the above conclusions.

ANALYSIS OF THE uPA SYSTEM IN MOUSE DEVELOPMENT.

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The processes of development and organogenesis involve remodeling of the extracellular matrix and cell migration, events which require targeted proteolysis. Proteases appear to play a major role in matrix modeling in tissue repair, angiogenesis, tumor development, processes that mimic events occurring during embryogenesis. The cellular localization of the various components involved in plasmin production (plasminogen, tissue-type plasminogen activator or tPA, urokinase-type plasminogen activator or uPA, uPA receptor), is suggestive of a function for these molecules in cell-surface mediated proteolysis. In order to elucidate the role of plasminogen activation during development, we have analyzed the expression of uPA, uPA receptor, and uPA inhibitors in mouse embryos from 4 to 14.5 days by in situ hybridization. During implantation, the domain of expression of each gene is strikingly well defined and precisely modulated. At later stages, uPA is found to be expressed in developing kidney , as well as in a pattern that appears to correspond to migrating neural crest cells.
Regulation of plasmin activity is mainly controlled at the level of PA and PA inhibitors, since circulating plasminogen does not appear to vary over a range of physiological states. In vitro, PA and PA inhibitors levels are influenced by several growth factors and hormones. To define a cause-and-effect relationship between growth factors and matrix-degrading proteases , the pattern of expression of the various components of the uPA system is analyzed in relationship to that of growth factors, as FGFs and TGFβs, which have been shown to modulate their expression in vitro.

A CONSERVED DOMAIN OF THE VENTRICULAR ZONE GENERATES OLIGODENDROCYTE PRECURSORS IN MAMMALS, BIRDS AND AMPHIBIANS.

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In the embryonic rat spinal cord, oligodendrocyte precursor cells can be identified *in situ* using RNA probes specific for the platelet derived growth factor alpha-receptor (PDGF-αR)[1,2] or the myelin protein 2',3'- cyclic nucleotide 3'-phosphodiesterase (CNP)[3]. Oligodendrocyte precursor cells originate in the ventral half of the cord around embryonic day 14 (E14). Initially they form discrete longitudinal columns of cells in the ventral ventricular zone on either side of the central canal. Subsequently, the oligodendrocyte precursor cells appear to migrate away from this region and disseminate throughout the spinal cord. We now have direct evidence for migration of quail oligodendrocyte progenitors in chick-quail chimeras.

We have examined spinal cords of *Xenopus* and chicken embryos and find that the distribution of oligodendrocyte precursors is similar to that seen in the rat.

In an attempt to understand how the formation of the original focus of oligodendrocyte precursors is controlled in the dorsoventral axis of the spinal cord, we have ablated the notochord in *Xenopus* embryos and find that no oligodendrocyte precursors appear in these animals. This implies that the notochord is important for controlling commitment to the oligodendrocyte lineage in the ventricular zone of the CNS.

We are currently investigating how the original focus of oligodendrocyte precursors is controlled in the dorsoventral axis of the spinal cord by grafting an ectopic notochord into a dorsolateral position relative to the spinal cord of chicken embryos.

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V. Neural Development II

ENVIRONMENTAL FACTORS IN NEURAL CREST CELL DIFFERENTIATION

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The neural crest is a pluripotent structure of the vertebrate embryo the cells of which differentiate after a phase of migration into a large variety of cell types. In vivo grafting experiments, taking advantage of the quail/chick marker system, allowed the fate map of the neural crest to be constructed and the precise level of origin along the neuraxis of the component cells of PNS ganglia and other neural crest derivatives to be precisely defined. It appeared that the neural crest is regionalised in different areas from which a limited number of phenotypes arise. However, if the position of neural crest fragments is experimentally changed prior to the onset of migration, it turns out that virtually all the cell types represented in PNS ganglia can arise from any level of the neural crest provided it is transplanted into the appropriate level of the neuraxis. This means that the microenvironment into which the neural crest cells migrate plays a critical role in directing their differentiation. Further in vivo studies along with in vitro clonal analysis of neural crest developmental potencies revealed that, during the migratory phase, the crest cells are already highly heterogeneous in their state of commitment. The existence of such diverse neural crest precursors suggests that they are lineally-related and therefore favors the hypothesis that crest-derived cell types are generated through step-wise restrictions of the developmental potentials of initially totipotent stem cells. The existence of such totipotent cells was demonstrated in the progeny of cephalic neural crest cells which yield not only neurons, glia and melanocytes but also mesectodermal derivatives such as cartilage and smooth muscle cells.

The analysis of the phenotypes represented in more than 500 clones derived from the migrating cephalic neural crest revealed that not all the possible cell type combinations were present. Precursors with the ability to generate cartilage and those capable

of giving rise to melanocytes seem to segregate early. By constrast, all bipotent cells retain a gliogenic potential, suggesting that commitment to every specific lineage possibly progresses through a bipotent glial + X precursor, X being either neuron, melanocyte or cartilage. Another feature coming out of these experiments was the demonstration that the pluripotentiality of neural crest-derived cells progressively decreases with increasing embryonic age. Trunk neural crest cells located in the sclerotomal moiety of the somites and non-neuronal cells of the developing sympathetic ganglia and dorsal root ganglia (DRG) were tested in this respect. Both the size of the clones and the diversity of the phenotypes they contained progressively decreased with the age of the donor embryo. The temporal restrictions of the differentiation potentials of cells derived from the rhombencephalic neural crest which colonize the gut were also examined in clonal cultures. Crest cells homing to E4 quail gizzard are able to generate clones containing both glial and neuronal cells, and others composed only of non-neuronal cells. However, the capacity of enteric neural crest-derived precursors to yield neurons disappears from E6 onwards.

These different studies showed that, immediately or soon after initiation of emigration from the neural tube, the neural crest appears as a mixed population of pluripotent cells together with cells restricted in their developmental capacities. The latter increase in proportion with progressing age of the embryos, leading to the selection in the various neural crest derivatives of those progenitors that are committed to phenotypes appropriate to these different sites. Coming back to in vivo development of neural crest derivatives, these results imply that cells which aggregate to form a ganglion are endowed with a diversified range of requirements for growth and survival. One can therefore assume that the final phenotypic choice made by a precursor cell, in a given site of neural crest differentiation, results from a cooperation between extrinsic factors of the microenvironment and cell-intrinsic properties that modify its responsiveness to external influences.

Next investigations concerned the nature of such influence. Data related to the differentiation of the adrenergic, melanocytic and glial cell lineages will be reviewed.

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CYTOKINES IN NEURAL DEVELOPMENT AND INJURY, Paul H. Patterson, Biology Division, California Institute of Technology, Pasadena, CA 91125, USA.

As in the hematopoietic system, the enormous variety of phenotypes in the nervous system arises, in part, through the action of instructive differentiation signals. Neuronal culture assays have allowed the identification and cloning of several proteins that control the expression of phenotype-specific genes in developing neurons. A group of non-homologous cytokines induces the expression of the same set of neurotransmitter synthetic enzymes and neuropeptides in cultured sympathetic neurons. Several members of this cytokine group, which now includes CDF/LIF, CNTF, OSM and GPA, share a predicted secondary structure, as well as the use of a transducing receptor subunit in common with IL-6 and IL-11. The latter two cytokines also display a weak activity in the sympathetic neuron assay. In addition, we find that certain members of the TGFβ superfamily, activin A and BMP-2 and -6, each induce a distinct set of genes in sympathetic neurons, and that set overlaps in part with the spectrum of neuropeptides induced by the neuropoietic family. Neuronal responses to these various cytokines are differentially regulated by depolarization, such that neuronal activity can modulate phenotypic expression in a cytokine- and neuropeptide-specific manner.

To study the role of CDF/LIF *in vivo*, we are localizing its mRNA and that of its receptor. CDF/LIF mRNA levels are developmentally modulated, and substantial differences are observed between tissues. Particularly high expression for both CDF/LIF and the LIFR is found in the visual system of the adult rat. In sciatic nerve and sympathetic ganglia, the postnatal increase in receptor expression parallels expression of CDF/LIF.

Analysis of mice in which the CDF/LIF gene has been targeted by homologous recombination reveals a role for this cytokine in the injury response as well as in the normal brain. CDF/LIF mRNA levels increase dramatically after damage to normal, mature peripheral nerve and ganglia, and a series of neuropeptide mRNAs are induced. This neuropeptide induction is not nearly so pronounced in the homozygous LIF$^-$ mutant mice, demonstrating a role for CDF/LIF in this injury response. In addition, the brains of the adult LIF$^-$ mutant mice display several atrophic changes in the visual cortex and dentate gyrous of the hippocampus. Similar changes are not observed in several other areas of cortex or other parts of the hippocampus.

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CONTROL OF CELL SURVIVAL BY NEUROTROPHINS

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During normal development of the vertebrate nervous system, many cells initially generated are eliminated when long lasting relationships are first established, for example when neurons contact their target cells, or oligodendrocytes their axons[1]. These cells seem to die by apoptosis, a program that can be blocked by specific, diffusible signals. Their limited availability presumably explains why cell death is observed during development. Some of these factors belong to the neurotrophin gene family, which comprises 4 members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). All neurotrophins prevent the death of a variety of *cultured* PNS or CNS neurons. NGF, BDNF and NT-3 are also needed in vivo if normal neuronal numbers are to be maintained in the PNS[2-7]. Presumably, the vertebrate PNS would lack entirely in the absence of these 3 genes. In addition, it is becoming apparent that NT-3 also acts very early during development, when neurons are still being generated. For example, already during the formation of peripheral sensory ganglia, the limited availability of NT-3 regulates neuronal proliferation and/or the differentiation of neuronal progenitor cells[7]. In addition, a direct mitogenic effect of NT-3 has been observed on oligodendrocyte precursor cells, and NT-3 is necessary in vivo to reach normal numbers of oligodendrocytes in the optic nerve[8]. Thus in the PNS, it appears that NT-3, like NGF and BDNF, control neuronal numbers, but that this control involves mechanisms operating before (and, most probably, in addition to) those described for NGF, i.e. the prevention of the death of neurons that have already sent their axons to their target cells.

The exact role played by the neurotrophins on CNS neurons in vivo is less well understood, and no single neurotrophin gene seems to be required for the survival of any CNS neuronal population. For example, in mice whose functional NGF or BDNF gene has been deleted by homologous recombination, no massive cell death (beyond that normally seen during development) could be observed in a series of neurotrophin-responsive structures, such as the basal forebrain cholinergic neurons, the spinal or facial motoneurons, the dopaminergic neurons of the substantia nigra, or the retinal ganglion cells[3,4,5]. This is intriguing, as these groups of CNS neurons show a response to neurotrophins either in vitro or after section (or lesion) of their axons in vivo. To obtain clues about potential roles of BDNF during normal development of CNS neurons, we studied the visual system of the chick. The optic

tectum represents the only major target of the retinal ganglion cells, and these structures can be manipulated during development. In addition, the timing of the arrival of the first retinal ganglion cell axons is known with precision. Finally, a previous study had established that in vitro, chick retinal ganglion cells respond to BDNF: when dissociated from E11 retinae, few will survive in the absence of BDNF[9]. This corresponds to the stage when normally occurring cell death starts to operate in vivo in the retinal ganglion cell layer. To study the expression of the BDNF gene, a simple, PCR-based quantification method was set up[10]. A mutated RNA standard was used that differs from the endogenous mRNA by a single base, thus creating a restriction site not present in the wild-type message. This allows the standard (added at the time of tissue homogenisation) to be distinguished from the endogenous mRNA at the end of the amplification procedure. This method, which involves quantification using a single reaction tube and low cycle numbers, was validated by comparing the copy numbers determined with those obtained with quantitative Northern blot analysis (performed with tissues containing relatively high copy numbers). BNDF mRNA levels was detected in the tectum already at E4, which is 2 days before the first retinal ganglion cell axons reach the tectum. This might be related to the development of the tecto-bulbar tract, which expresses high levels of trkB (a BDNF receptor) at this stage. At E6, the lowest BDNF mRNA levels were determined. Only 300,000 copies per mg tissue were found, and assuming that the tectum has a cellular density similar to that found in the retina, for which cell numbers are available, this would correspond to about one copy of BDNF mRNA for every 5 cells. A strong increase (of more than 10-fold) follows until E11, and thereafter the levels decrease by about 30% until E17. We were interested to see if the massive increase in copy numbers seen in the tectum after E6 can be correlated with the arrival of the retinal ganglion cell axons. In order to explore this possibility, the right optic stalk was severed at E4 to prevent tectal innervation. At E7, the left, non-innervated tectal was compared with the innervated side. A reduction by 43% in the operated side was observed when compared with the contralateral, non-operated side. In order to test for a possible role of electrical activity, tetrodotoxin was injected into one eye at E6. Comparison of BDNF mRNA levels in both tecta at E7 showed a reduction in the tectum contralateral to the injected side by 32%. This suggests that a significant portion of the effects resulting from the absence of tectal innervation can be accounted for by action potential-mediated events taking place in the retinal ganglion cells. These results indicate that the first projecting ganglion cells are electrically active, and that this activity has measurable consequences on the steady-state levels of BDNF mRNA in CNS target cells. Thus, it appears likely that the neurotransmitter(s) released by the electrically active ganglion cells play a role in the regulation of BDNF levels. However, in view of recent results obtained with BDNF -/- mice[4,5], it appears unlikely that a major function of BDNF would be to

regulate the survival of ganglion cells in the retina during normal development. Amongst possibilities for the function of BDNF might be the regulation of branching of the axon terminals, of the motility of the growth on the tectum, of the release of neurotransmitters or of the expression of various neurotrasmitters by the ganglion cells.

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Controlling Oligodendrocyte Numbers

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We have studied the cell-cell interactions that control cell numbers in the oligodendrocyte cell lineage in the developing rat optic nerve. The final number of oligodendrocytes in the optic nerve depends on the number of precursor cells that migrate into the nerve, the number of divisions that the precursors undergo before differentiating, and the number of newly formed oligodendrocytes that undergo programmed cell death(PCD).

The number of precursor cell divisions depends on an intrinsic clock in the precursor cell that consists of at least two components: a counting mechanism that is driven by growth factors such as PDGF, NT-3 and IGFs, and an effector mechanism that stops cell division and depends on extracellular hydrophobic signals such as thyroid hormone.The proliferation of the precursor cells in vivo depends on electrical activity in the axons in the nerve.

Fifty percent of newly formed oligodendrocytes die during normal development, apparently because they fail to get sufficient stimulation by extracellular survival factors that the cells seem to need to avoid PCD. PDGF, NT-3, IGF-1 and CNTF all support the survival of newly formed oligodendrocytes in vitro and suppress their death in vivo.Oligodendrocyte survival in the developing nerve depends on axons, but in this case electrical activity is not required.

We suspect that cell number control operates in much the same way for many types of vertebrate cells, and that all such cells are programmed to kill themselves unless they are continuously signalled by other cells not to do so.

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POSTERS

BRAIN-DERIVED NEUROTROPHIC FACTOR AND ITS RECEPTORS IN THE DEVELOPMENT OF THE CHIKEN RETINA

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Receptors for brain-derived neurotrophic factor (BDNF, BDNFR) were characterized in the retina between embryonic days (E)5 and 14. At any age, cells displayed specific high-affinity binding for ^{125}I-BDNF. The number of BDNFRs per cell varies along development. An early phase of BDNFR binding occurred at E5-7, coincidental with the main onset of neuron differentiation. BDNF binding then transiently decreased at E8-10 to reach a second maximum later, on day E14. Kinetic analysis indicates that early and later expressed BDNFRs are different from each other. By selective immunoprecipitation, E14 BDNFRs were identified as trkB tyrosine kinase of 140 kDa. E7 receptors displayed a trk-like immunoreactivity, but were immunoprecipitated as a 110-kDa band. Northern Blot analysis showed only one 9.5 kb transcript of *trk*B, which also appeared in two successive phases. In general, the developmental profile of *trk*B expression paralleled that of ^{125}I-BDNF binding. In situ hybridization of E7 retinas revealed that *trk*B message was distributed throughout the undifferentiated retina. As differentiation proceeded, trkB expression became exclusively associated to the ganglion cell layer. Accordingly, binding autoradiography with ^{125}I-BDNF revealed specific BDNF receptors on cell bodies of the ganglion cell layer and dendritic processes extending into the inner plexiform layer.

The two types of BDNFR likely differ in their functional roles. BDNFR expressed at younger ages was found to potentiate the differentiating action of NT-3 and to inhibit, again in combination with NT-3, mitosis of proliferating neuroepithelial cells. Later in development, BDNF supports the survival and maturation of differentiated retinal ganglion cells (Rodríguez-Tébar et al., *Dev. Biol.* 136, 296-303, 1989). We propose that BDNF binds to two distinct classes of specific, developmentally regulated trkB receptor variants that mediate different signals in accordance with the changing needs of developing cells.

Expression of the epidermal growth factor (EGF) gene during mouse development

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EGF stimulates the growth or differentiation of a variety of cells through activation of the EGF reecptor at the cell surface. Mature EGF (53 amino acids) is derived from an unusually large precursor of 1217 amino acids. To gain insight to the involvement of EGF/EGF precursor during mammalian development, we have studied the expression of the EGF gene in day 7.5 - 14.5 mouse embryos by whole mount *in situ* hybridization. EGF transcripts can be detected in gonads (day 12 - 14.5), lungs and adrenal glands (day 13.5 - 14.5). Expression of the EGF receptor gene cannot be detected using the same procedure. Immuno-histochemistry studies using an anti-body to mouse EGF detected weak expression of EGF in the developing gonad (day 12.5 - 14.5), adrenal gland, and stronger expression in metanephric tubules (day 13.5- 14.5). For day 14.5 embryos, the lower limb was also included in the study. Expression of EGF can be detected in bone and cartilage.

ROLE OF NODAL IN MOUSE GASTRULATION

J. Collignon

Analysis of a retrovirally-associated recessive lethal mutation, termed *413.d*, has allowed the identification and cloning of a gene representing a promising candidate for controlling mesoderm formation in the mouse embryo[1]. The *413.d* mutant phenotype is characterized by growth retardation, disorganization of ectodermal cells and absence of a primitive streak[2]. Chimera studies have shown that the mutation functions in a non cell autonomous fashion[2]. Molecular characterization of the *413.d* locus allowed the identification of two independent transcription units, both of which appear to span the retroviral insertion. The gene mapping 3' to the virus encodes a novel molecule, the potential function of which remains unclear from sequence analysis of corresponding cDNA clones. Preliminary experiments have indicated that this gene is expressed throughout development and in all tissues of the adult animal. The transcription unit lying 5' to the virus encodes a novel member of the TGF-ß superfamily of secreted growth factors, now called *nodal*. *In situ* hybridization has shown that the *nodal* mRNA is initially detected at day 5.5 in a gradient in the early embryonic ectoderm. *Nodal* RNA is also expressed in the primitive endoderm overlying the primitive streak. At the end of gastrulation *nodal* expression becomes confined to a small population of cells surrounding the anterior tip of the primitive streak. This region, termed the node, is thought to be analogous to the blastopore lip of *Xenopus* and Hensen's node in chick, both of which have been shown experimentally to induce secondary embryonic axes and thus possess "organizer" activities[3,4]. As one of the general hallmarks of *413.d* mutant embryos is an apparent failure to form definitive mesoderm, the expression pattern of the *nodal* gene is consistent with the notion that it has a role in gastrulation. Nonetheles, about 25% of mutant embryos do form randomly positioned patches of cells of a posterior mesodermal character (i. e, expressing either *brachyury* and/or *Cdx-4*). No marker of anterior mesoderm was found to be expressed in these embryos.

Our current interest is to assess the exact role of *nodal* during gastrulation. Does it induce mesoderm? does it play a role in the patterning of mesoderm? Is it necessary to induce the migratory behaviour of cells from the primitive streak? Is it a trophic factor for cells from the streak? and since the phenotype seen in *413.d* mutant embryos occurs later than the onset of nodal expression in wild type embryos, when and where is the gene product truly required for gastrulation to proceed? We are now looking at these questions in mouse, *Xenopus* and chick.

The fact that a minority of mutant embryos do express posterior mesodermal markers could result from *413.d* being a leaky mutation. To address this possibility we are generating a null allele of *nodal* by gene targeting. We have also obtained anti-nodal antibodies to investigate the distribution of nodal protein in both wild-type and *413.d* mutant embryos. Preliminary results indicate that mouse *nodal* RNA can induce mesoderm formation in *Xenopus* animal caps when injected in *Xenopus* eggs.

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TGF-α-LIKE PROTEIN IN THE CHICKEN KIDNEY

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Transforming Growth Factor-α (TGF-α) is a polypeptide related to EGF. Both bind to EGF-Receptor (EGF-R) to carry out their function. We have identified a TGF-α-like protein in the chicken kidney by immunohistochemistry and Western blot analisys. Chicken kidneys from embryos and adult animals were fixed in Bouin, dehydrated, embedded in paraffin and sectioned. We used a monoclonal anti-human TGF-α and the immunoperoxidase technique, and counterstained sections with alcian blue or PAS. The immunoreactive cells for TGF-α were localized in the distal tubules of the nephrons (alcian blue and PAS negative tubules) and showed an alternating pattern of distribution in the tubules. Scarced cells of the collecting tubules were also immunostained. Immunoreactivity was mainly located in the cytoplasm. We also found immunoreactivity for EGF-R in the proximal tubules of chicken embryos.

Kidneys from postnatal chickens and rats were homogenized. Cytosolic, membrane extracted and membranous fractions were collected and proteins were separated by the tricine-SDS-PAGE system and transfered to immobilon P membranes. Using two monoclonal anti-TGF-α antibodies we detected a protein of apparent molecular weight of 14, 31 and 45 Kda. These proteins are sensitive to the action of elastase, releasing different peptides which also include the TGF-α epitope.

These results suggest that a protein with a TGF-α epitope may be involved in the differentiation mechanisms of the chicken kidney by binding to EGF-R.

Substance P Modulates Chemotropic Influences of Floor Plate on Developing Neurites in the Central Nervous System. C De Felipe, R.D.Pinnock° and S. P Hunt. (*Spon. BRA) MRC Laboratory of Molecular Biology, Division of Neurobiology, Hills Road, Cambridge, CB2 2QH, °Parke-Davis Neuroscience Research Unit, Robinson Way, Cambridge, UK CB2 2QB.

The floor plate, a neuro-epithelial structure found at the ventral midline of the developing central nervous system, has been shown to function in dorso-ventral patterning in the spinal cord and at later embryonic stages, in guidance of commissural axons by release of chemoattractants. We have found that embryonic rat floor plate cells express the neurokinin 1 receptor and that there is a transiently appearing subpopulation of commissural axons containing Substance P, the neuropeptide ligand for this receptor. Receptor coupling to an intracellular effector system in dissociated floor plate cells was established by monitoring the intracellular calcium levels of dissociated floor plate cells using the Ca^{+2} indicator fura-2. Bath application of 10 to 1000nM substance P or the selective NK1 receptor agonist [Sar^{9},$Met(O_2)^{11}$]substance P^{12} for 120s produced a rapid and reversible increase in internal calcium levels which was concentration dependent. Prolonged application of sub-threshold concentrations (0.1-3nM) of substance P applied for up to 10min produced rhythmic oscillations in internal calcium levels. The NK2 and NK3 receptor agonists [β-Ala^{8}]neurokinin A-(4-10) and senktide (100nm) were inactive. 50nM concentrations of the rat selective NK1 receptor antagonist RP67580 blocked the response to substance P. Finally, Substance P analogues increased the amount of axon outgrowth from dorsal horn explants when co-cultured with floor plate in collagen gels. These results suggest that SP released from pioneering neuronal pathways can subtly change the extracellular environment and influence the growth and differentiation of developing neuronal systems by regulating the release of chemoattractants from floor plate cells.

An interaction between odd-numbered rhombomeres and their neighbours controls their depleltion of neural crest cells

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Hindbrain neural crest are segregated into three separate morphogentically specified streams of cells. These three streams originate at discontinuous levels along the rostrocaudal axis of the rhombencephalon, centred on rhombomeres 1 and 2, 4 and 6 respectively. These are separated by rhombomeres r3 and r5 which are depleted of neural crest cells. Here we show that the regions of r3 and r5 expected to produce neural crest are associated with elevated levels of programmed cell death and as such act to separate each of the 3 morphogenetically specified streams of hindbrain crest. These regions are also marked by their expression of members of the *msx* family of homeobox genes with *msx 2* expression preceding programmed cell death in a precisely co-localised pattern. *In vitro* and *in vivo* experiments have revealed that r3 and r5 are depleted of neural crest cells by an interaction within the neural epithelium: if isolated or distanced from their normal juxtaposition, both r3 and r5 will produce neural crest cells. When r3 and r5 are unconstrained in this way allowing production of neural crest , *msx 2* expression is concomitantly down regulated. This suggests an intimate relationship between *msx 2* expression and programmed cell death in this system. It has been demonstrated that BMP 4 induces *msx* gene expression during odontogenesis. Our *in situ* analysis has demonstrated that it's spatial and temporal deployment is consistent with it playing a similar role in this system. Moreover the addition of recombinant BMP 4 to rhombomere explants results in the specific depletion of neural crest from r3 and r5.

IN SITU GENE EXPRESSION PATTERNS OF IGF TYPE 1 RECEPTOR AND IGF LIGANDS DURING AVIAN EMBRYONIC DEVELOPMENT

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We have studied Insulin-like Growth Factor 1 (IGF1) and IGF2, as well as IGF type 1 receptor (IGF-R) gene expression patterns during avian embryonic development by means of reverse transcriptase-PCR and in situ hybridization (ISH) with chicken specific probes. We cloned and sequenced chicken IGF type 1 receptor from cDNA. Detection of IGF-R mRNA by ISH in early embryos (from E2 onwards) revealed high transcript levels in the neuroepithelium, notochord and spinal ganglia, as well as in several peripheral structures including branchial arches, mesonephros, lung primordia and limb buds. RT-PCR amplification of IGF1 message levels revealed the presence of specific transcripts from the 10-somite stage onwards, but no signal could be obtained by ISH before E12. At later stages (E14-E16) we compared the ISH patterns of IGF-R, IGF2 and IGF1 gene expression. In tectum and cerebellum IGF-R gene expression was widely distributed, while IGF2 mRNA was restricted to the Purkinje cell layer. The distribution of IGF-1 transcripts was similar to that of IGF-R. Both ligand genes were strongly expressed in some thalamus and brainstem nuclei. IGF1, IGF2 and IGF-R genes were expressed in neuroretina, each showing a particular pattern. In large areas of the telencephalon, including the mitral cell layers of the olfactory bulbs, cells accumulated receptor and IGF1 mRNAs. According to CNS regions these results suggest an autocrine or paracrine mode of IGF1 action on embryonic neurons.

Expression patterns of aFGF, FGF-R1 and FGF-R2 in the developing chick retina and effects of exogenous FGFs on growth, differentiation, and survival of chick neural retina cells.

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Fibroblast Growth Factors (FGFs) constitute a family of nine mitogenic polypeptides having a high affinity for heparin. The two prototypes well known members of the family : acidic and basic FGFs (a and b FGFs) are signalling molecules implicated in a wide variety of biological processes such as cell growth, differentiation and survival. They have been purified from retina. We first reported which cells in the chick retina expressed aFGF at the different stages of embryonic and post-hatching development. The specific aFGF mRNA was detected by in situ hybridization employing riboprobes in all neuronal layers with a timing corresponding to their sequential differentiation, starting in the ganglion cells and finishing in the photoreceptor nuclei layer.

FGFs act by high affinity receptors with tyrosine-kinase activity. To date five FGF receptor genes have been cloned. We initially focused our attention on the expression of FGF-R1 and R2 receptors using Northern blot and PCR analysis. Next, we studied the same expressions at a cellular level using in situ hybridization (1). They follows the retinal layering according to a gradient of differentiation from the optic nerve to the cilliary process and from the vitreous humor to the choroid.

Embryonic chick neural retina cells (NR) differentiate in vivo into neurons and glial cells. They also show a remarkable capacity to transdifferentiate into lentoid bodies and pigmented cells in prolonged culture. Chick embryo NR cells were used in vitro to examine the effects of exogenous a and b FGFs, on proliferation and protein accumulation (2).

We demonstrate that the rate of proliferation varies over the period of culture with a maximum occuring after two weeks, followed by a decrease concommitant with the appearance of lentoid bodies. Both factors increase the proliferation of glial cells, stimulate the differentiation of the photoreceptors and modulate the survival of neurons without affecting protein accumulation within these cells. The concentration of aFGF was measured using an enzyme immuno-assay and showed an accumulation of this protein only in bFGF treated cultures, suggesting that bFGF positively modulates aFGF synthesis in neural retina cell cultures.

1. M. Tcheng, G. Fuhrmann, M.P. Hartmann, Y. Courtois and J.C. Jeanny
Exp. Eye Res., in press.
2. M. Tcheng, L. Oliver, Y. Courtois and J.C. Jeanny
Exp. Cell Res., in press.

Development, degeneration, and tumorigenesis of myelin-forming glia

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Myelin is a membrane characteristic of nervous tissue, laid down in segments along selected nerve fibers, that functions as an insulator to increase the velocity of stimuli being transmitted between a nerve-cell body and its targets. In the CNS, oligodendrocytes are responsible for the formation and maintenance of myelin and a single oligodendrocyte may maintain as many as 30-50 internodes of myelin. In contrast, Schwann cells, the myelin-forming cell of the PNS, elaborate internodal myelin at a 1:1 ratio. Oligodendrocytes originate from precursor cells in the ventricular zone of the neural tube, whereas Schwann cells are derived from migrating neural crest derivatives. The mitotic potential of myelin-forming oligodendrocytes and Schwann cells is very different and has implications in disease and reparative processes. The remyelinating capacity of the Schwann cell is based on the cell's ability to degrade myelin and resume cell proliferation. In contrast, only limited remyelination of myelin-depleted axons in the CNS is observed. Terminally differentiated oligodendrocytes are post mitotic and thus actively regulated not to enter the mitotic cycle. Recent experiments by myself and collaborators showed that SV40 large T-antigen expression in differentiated oligodendrocytes in transgenic mice was associated with ablation of myelin-forming oligodendrocytes from the white matter. Thus, deregulated cell cycle activity in post mitotic oligodendrocytes seems to trigger a degenerative response (probably by apoptosis) as opposed to a preneoplastic (hyperproliferative) response. Major differences in cell cycle and apoptosis regulation in differentiated oligodendrocytes and Schwann cells may explain why oligodendrogliomas are very rare tumors of the CNS whereas malignant Schwannomas are major PNS tumors.

Transgenic mouse models and transgenic mouse-derived glial cell lines are used to investigate the pathogenic potential of dominant cell cycle regulatory genes and POU-domain transcription factors in differentiated oligodendrocytes and Schwann cells. In addition, subtractive molecular cloning techniques are employed in the identification of genes differentially expressed during cAMP stimulation of a transgenic mouse-derived oligodendrocyte and Schwann cell line.

INVOLVEMENT OF NEUROTROPHINS IN THE PHENOTYPIC SPECIFICATION OF CHICK CUTANEOUS AFFERENTS:

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Primary afferents critically depend on neurotrophins for survival during embryonic development, however it has recently become clear that neurotrophins may also influence the phenotypic fate of sensory neurons during development (Lewin et al. 1993 J.Neurosci. 12:1896-1905). Sensory neurons in the DRG of the chick embryo have been commonly used as a model to examine the effects of neurotrophin deprivation or addtion during development. Here we have developed a technique to study the functional properties of individual sensory neurons in the chick in order to address this question. To this end the cutaneous femoralis medialis (CFM) nerve was dissected from late stage chicks (E17 onwards) together with the skin of its innervation territory on the medial thigh. This preparation was placed in an organ bath and recordings were then made from single primary afferents with mechanical receptive fields in the skin using standard teased fibre techniques. With this method primary afferents could be classified according to their conduction velocity, mechanical thresholds, thermal and chemical sensitivity. In normal untreated embryos neurons were found to have response properties appropriate to their conduction velocities. Thus slowly conducting afferents (<1.0 m/s) could be classified as nociceptors responding to noxious mechanical, thermal, and chemical stimuli or various combinations of these. On the other hand faster conducting afferents had lower mechanical thresholds and did not respond to noxious stimuli such as heat or chemicals. We have gone on to use this model to examine the effect manipulating the levels of neurotrophins *in ovo*. To do this we injected cultured cells onto the chorio-allantoic membrane of embryos at E3 which secrete neurotrophins (NT-3) or specific monoclonal anti-bodies to the neurotrophins (anti-NT3 and Anti-NGF). At E17 or later recorded from single neurons innervating the skin in the treated embryos. Preliminary results indicate that by depriving animals of NGF during development neurons responding specifically to noxious heat are removed in the chick embryo as this type was not observed after anti-NGF treatment. Interestingly, with excess NT-3 treatment from E3 the mechanical thresholds of specific types of cutaneous afferents appeared to be lowered. Thus results so far indicate that the phenotypic development of sensory neurons in the chick embryo may be influenced by the availabilty of neurotrophins. Further experiments may now be designed to ascertain more precisely when in development these neurotrophins can exert these effects.

NEURALIZING FACTOR FROM EMBRYONIC CHICK BRAIN: BIOCHEMICAL CHARACTERISTICS AND IN VITRO EFFECTS ON EARLY GASTRULA ECTODERM OF DIFFERENT AMPHIBIAN SPECIES.

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We have addressed questions of neural competence in amphibian early gastrula ectoderm using an explant culture technique and neuralizing factor isolated from forebrain of 7-8 day old chick embryos. This factor appears to belong to soluble heparin-binding glycoproteins (Mikhailov et al. Russian J. Devl. Biol. 1993. V.24. N2, P. 24-32). We have tested the neuralizing action of chromatographic fraction, containing this factor, on animal pole ectoderm (APE) isolated from early gastrulae of *Rana temporaria*, *Triturus alpestris*, and *Xenopus laevis*. APE explants of different species were characterized by "selective sensitivity" to neuralizing influence of this fraction. We have found the induction of neural tissue in *R. temporaria* (neuralization up to 100% cases) and *T. alpestris* (neuralization up to 60% cases) explants, but no neuralization was detected in APE cultures of *X. laevis*. These results could be observed in the explants treated with different concentrations of neuralizing factor for different periods of time. Control experiments have demonstrated that APE of studied amphibian species is resistant to "autoneuralization" in vitro.

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CHOLINERGIC SIGNALLING IN HEMATOPOIETIC DIFFERENTIATION

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Evidence is accumulating that cholinergic signalling is involved in the differentiation of hematopoietic stem cells (HSC) in the bone marrow (BM). Using phosphorothioated antisense oligodeoxynucleotides(AS-oligos) to the AUG initiator region of the mRNA encoding butyrylcholinesterase (BCHE) we found that megakaryocyte (MK) maturation, induced by interleukin-3 was blocked, resulting in a 40-50% decline in MK colony (CFU-MK) production. This was accompanied by a distinct shift in hematopoietic differentiation as a majority of the surviving HSC were diverted to mononuclear cell formation. We then employed antisense inhibition to examine the hematopoietic functions of a novel gene, CHED, exhibiting a sequence homology to the cell division controlling (cdc) kinases(Lapidot-Lifson et al, PNAS 89:579,1992). AS-CHED proved to be lineage-specific in action only on MK proliferation as opposed to the general controller, cdc2Hs, which interfered non-specifically with cell division on all hematopoietic cells. Using RNA-PCR amplification of cDNA reverse-transcribed from cellular mRNA, it was found that AS-CHED was incorporated into the cells and selectively destroyed its target mRNA but not AS- or S-BCHE-treated cells. In parallel RNA-PCR experiments with primers for actin, known to increase four-fold during MK maturation, the CHED/actin mRNA ratio was sharply reduced from control values within one hour and remained low for four days, demonstrating the rapid and stable activity of the AS-oligo. CHED/actin mRNA ratios increased with time in both control and AS-CHED-treated cultures, implying that AS-CHED acts only at the post-transcriptional level. Sequence analysis of BCHE and acetylcholinesterase(ACHE), a well-known marker of MKs, indicated that both the ACHE and BCHE proteins possess S/T-P-X-Z motifs, common to cdc kinase sustrates. It is suggested, therefore, that all three of the genes examined, BCHE, ACHE and CHED, are interrelated in their action on hematopoietic differentiation by a mechanism to be clarified.

IMMUNOHISTOCHEMICHAL LOCALIZATION OF TGF-α IN RAT COLON

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Transforming growth factor α (TGF-α) is a 50-amino acid polypeptide wihch has been related to cell proliferation and differentiation. Proximal and distal colon from fetal, newborn and adult rats were studied by immunohistochemical techniques using a monoclonal antibody against human and rat TGF-α. Immunoreactive TGF-α (IR-TGF-α) first appeared in distal colon at 18 days of gestation when the proximal colon remained negative. At all ages studied, the staining for TGF-α at the base of the crypts in the distal colon showed a supranuclear pattern. At 22 days of gestation and until 9 days of postnatal development, the proximal colon is negative for TGF-α. From day 10 to 24 of postnatal development IR-TGF-α cells with a cytoplasmic staining were confined to the lower half of the villi. Afterwards, cells at the crypts showed supranuclear immunostaining in the cis and trans cisternae of the Golgi apparatus and cells in the surface epithelium a cytoplasmic immoreaction in the polyribosomes. These results suggest a functional role for TGF-α in the establishment and maintenance of proliferation and differentiation during development.

REGENERATION OF DAMAGED DORSAL ROOT AXONS INTO THE SPINAL CORD IS PROMOTED BY ENSHEATHING GLIA FROM ADULT RAT OLFACTORY BULB.

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The unique regenerative properties of olfactory axons during the adulthood, could be due to the presence of ensheathing glia in the olfactory bulb (OB). To test whether these glial cells could be used to promote axonal regeneration we transplanted suspensions of pure ensheathing cells into adult rhizotomied spinal cords. Three morphologically, immunocytochemically and ultrastructurally different cell types could be identified in cultures from adult rat olfactory bulb. Out of those cell types, only p75-NGF receptor immunoreactive cells were ensheathing cells. This cell type was purified away from the others by immunoaffinity using anti-p75-NGF receptor. After laminectomy at the lower thoracic level, the spinal cord was exposed and one dorsal root was completely transected at the cord entry point. The root stump was microsurgically anastomosed to the cord and a suspension of purified ensheathing cells was transplanted in the spinal cord at the dorsal root entry zone. Three weeks after transplantation, numerous regenerating dorsal root axons were observed growing into the spinal cord. Regenerating dorsal root axons were labelled with DiI and with antibodies against CGRP and GAP-43. Primary sensory afferents, invaded laminae 1, 2 and 3, grew through laminae 4 and 5 and reached the dorsal grey commissure and lamina 4 of the contralateral side. We did not observe regenerating axons within the ipsilateral ventral horn and dorsal column. Transplanted ensheathing cells reached the same laminae as axons did. Neither ensheathing cells nor regenerating axons invaded those laminae they do not inervate under normal circumstances. In conclusion, the regeneration of injured dorsal root axons into the adult spinal cord was possible after ensheathing glia transplantation. The use of ensheathing cells as stimulators of axonal growth might be generalized to other CNS injuries.

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The expression of the 'G*ret*' receptor tyrosine kinase during early chicken embryogenesis, and its regulation by retinoic acid.

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Receptor tyrosine kinase genes (RTK) were cloned from chicken embryo neural tubes in order to identify receptors which may be involved in patterning the central nervous system.

One clone, was identified as the chicken homologue of the human Ret *proto*-oncogene, which is closely related to the PDGF, FGF and CSF receptor families. The ligand for RET is not known. We have made a temporal study of chicken *ret* (G*ret*) mRNA expression in early embryos, between Hamburger and Hamilton stage 5 and stage 20. It is expressed transiently in the neural tube (until stage 11) with a posterior limit of expression at the level of somites 6 and 7, and an anterior limit of expression in the hind brain, at the level of the boundary between rhombomeres 5 and 6. It is this region of the neural tube from which the vagal neural crest is derived. Most of the enteric neurones originate from the vagal crest, and these are absent in ret deficient transgenic mice. (V. Pachnis, personal communication). G*ret* transcripts are also expressed in the anterior half of the four most recently cleaved somites, as well as the presumptive somite which is about to cleave from the segmental plate. A high level of expression is detected in the intermediate mesoderm, which gives rise to the kidneys. At later stages of development there is a high level of G*ret* expression in some neural crest derived structures, such as the VII, IX and X ganglia, and in crest derived cells in the branchial arches. Our data suggest that ret has a role in the regionalisation of the somites and neural tube, and in the differentiation of some neural crest derived cell types.

Retinoic acid treatment of murine embryos has been shown to disrupt the molecular patterning of the hindbrain, and the axial limit of retinoic acid production in the normal mouse embryo lies in the posterior hindbrain region. We have demonstrated that acute treatment of cultured, stage 10, chicken embryos with retinoic acid induces G*ret* expression in more anterior regions of the hind brain.

Insulin-related growth factors are expressed in the developing chicken neuroretina and stimulate proliferation and differentiation of neuroepithelial cells. ***Enrique J. de la Rosa, Catalina Hernández-Sánchez, Ana López-Carranza, Mario García de Lacoba and Flora de Pablo. Centro de Investigaciones Biológicas C.S.I.C. Velázquez 144, E-28006 Madrid, Spain.***

Early neurogenesis progresses by an initial massive proliferation of neuroepithelial cells followed by progressive and sequential differentiation of the various mature neural cell types. We have studied the involvement of the insulin-related growth factors in these processes. E6-E8 neuroretina expressed both, preproinsulin and IGF-I mRNAs, as well as their respective receptor mRNAs, as demonstrated by RT-PCR. While insulin receptor and IGF-I receptor expression levels were relatively constant, the expression of the ligands mRNAs was developmentally regulated. Insulin-immunoreactivity with the characteristic mobility of proinsulin was found by extraction and HPLC fractionation of the vitreous humor. Therefore, the signaling elements of the insulin-related growth factor system are present during early neurogenesis and the retina, avascular at E6, may represent a paracrine model, including source and target, for this system. We used an organotypic neuroretina culture system to analyze the effects of the factors. Insulin and proinsulin showed close dose-response curves, with a plateau at 10^{-8} M, in stimulation of the proliferation of neuroepithelial cells, as determined by [^{3}H]-methyl thymidine incorporation. IGF-I was slightly more potent at low concentrations. Neural differentiation, as evaluated by the expression of the neuron-specific antigen G4, was stimulated to similar extent by insulin and IGF-I at 10^{-8} M and, slightly less, by proinsulin. Intravitreal injection of peptides *in vivo* confirmed the effect on differentiation of these factors. Ligand-binding studies with E6 retinal membranes revealed the presence of high affinity binding sites for IGF-I, insulin and proinsulin. Insulin-related growth factors, including proinsulin, are found in the developing chicken retina and may play a general stimulatory role in the process of neurogenesis, which, however, does not rule out other more specific cell-type functions.

TGF-βs: a role in Schwann cell developement?. Helen J. S. Stewart, Genevieve Rougon£, Kristjan R. Jessen and Rhona Mirsky. Dept. of Anatomy, University College, Gower Street, London WC1E 6BT, UK. £ CNRS Laboratoire de Genetique et Physiologie du Developpement, Universite d'Aix, Marseille II, 13288 Marseille Cedex 09, France.

The transforming growth factors - β are a family of growth factors implicated in many aspects of mammalian development. In this study we have examined the ability of these factors to influence Schwann cell development and differentiation. Using immunocytochemical techniques and Western blotting experiments we show that in vitro TGF-βs do not stimulate Schwann cells to express myelin proteins or lipids but do stimulate the expression of NCAM a protein expressed by precursor, embryonic and mature non-myelin-forming Schwann cells. Other Schwann cell phenotypic markers such as S100, glial fibrillary acidic protein and the p75 LNGF-R were also unaffected by TGF-βs. In other experiments we found that in vitro TGF-βs can potently inhibit cAMP mediated differentiation of Schwann cells towards a myelin related phenotype. TGF-βs prevented cAMP induced galactocerebroside, 04 antigen and myelin protein Po expression. Our studies also show that application of TGF-βs to Schwann cell cultures causes cell clumping and retraction resulting in a cell morphology resembling that of precursor Schwann cells. This is particularly apparent in cultures treated simultaneously with forskolin to elevate cAMP levels. Further experiments show that TGF-βs mRNA and protein are widely expressed in the developing peripheral nerve in both neurones and glia and are present in these cell types in culture. In vitro however Schwann cell TGF-βs are secreted in a latent form illustrating that the presence of TGF-βs does not necessarily reflect activity.

In summary, our results suggest that TGF-βs are able to dramatically influence Schwann cell phenotype in vitro. Furthermore, many (but not all) aspects of this effect suggest that TGF-βs many act as negative regulators of Schwann cell development in vivo.

Effects of ectopic expression of tissue specific transcription factors in *Xenopus laevis* embryogenesis

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To investigate the mechanisms and factors involved in differentiation processes during embryogenesis we introduce the tissue specific transcription factors LFB1 (also called HNF1) and HNF4 into fertilized eggs of *Xenopus laevis*.
These transcription factors, predominantly expressed in liver and kidney, might be involved in organogenesis as well as in establishment and maintenance of differentiation.
It is known that LFB1 mRNA is detectable in *Xenopus* embryos from the early gastrula stage on and therefore LFB1 is one of the earliest activated genes in embryogenesis. LFB1 protein expression is restricted to the middle part of the embryo and this part of the body will develop into liver, kidney, stomach and intestine, the organs expressing LFB1 in the adult animals (1). We induce an artificial expression of LFB1 protein in the head and the tail of the developing larvae upon injection of HNF4 mRNA into fertilized eggs. This ectopic induction of LFB1 might simulate processes normally occuring in the middle part of the embryo and reflects the fact that the LFB1 promotor contains a functional HNF4 binding site (2). However these embryos develop phenotypically normal, a phenomen that is also observed in embryos injected directly with LFB1 mRNA. As XDCoH, the *Xenopus* homologue of the LFB1 specific mammalian transregulator, might be essential for the LFB1 function, we cloned and functionally characterized the corresponding *Xenopus* cDNA. Using antibodies raised against the recombinant protein we detect small amounts of maternal XDCoH and a dramatic increase during organogenesis. Nuclei of tissues containing LFB1, such as the pronephros as well as the developing liver and intestine, do also express XDCoH. We are now analyzing embryos coinjected with LFB1 and XDCoH mRNA on the molecular level for the putative abnomal expression of LFB1 dependent genes.
As activin A in combination with retinoic acid leads to frequent induction of pronephric tubules in isolated animal caps (3), we analyzed the appearance of LFB1 in such explants. Clearly the combined treatment with activin A and retinoic acid induces LFB1 restricted to the nuclei of the pronephric tubules, whereas activin A or retinoic acid alone does neither induce pronephric tissue nor LFB1. Animal caps derived from HNF4 injected eggs produce LFB1 but no pronephric tubules in the absence of activin A and retinoic acid and we therefore conclude that these inducers do not simply activate HNF4 in animal caps.

(1) Bartkowski et al. (1993) Mol. Cell. Biol. 13, 421-431
(2) Zapp et al. (1993) Mol. Cell. Biol. 13, 6416-6426
(3) Moriy et al. (1993) Develop. Growth & Diff. 35 (2), 123-128

FUNCTIONAL EFFECTS AND BINDING PROPERTIES OF INSULIN-LIKE GROWTH FACTOR-I IN THE EARLY DEVELOPING EAR

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The role of insulin-like growth factors (IGFs) during the early development of the inner ear was investigated. IGF-I induced growth of otic vesicles that were isolated and cultured in vitro. IGF-I induced DNA-synthesis and increased cell number and mitotic rate, these effects were dose dependent, half-maximal concentration being 10^{-10} M. IGF-II was also able to induce growth but with a lower potency (maximal effect 10^{-8}-10^{-7} M). IGF-I induced otic vesicles to develop from stage 18 to stage 21 in a period of 24 h, thus mimicking normal mitotic pattern and morphogenesis in vivo. IGF-I also stimulated growth in the associated cochleo-vestibular ganglion (CVG) in a dose-dependent manner (10^{-10}-10^{-9} M). The study of the presence of the elements of the IGF system indicated that binding of ^{125}I-IGF-I to specific receptors occurred with high affinity. An autoradiographic study of sections from otic vesicles showed the location of labelled IGF-I in the epithelium, more densely grouped in the ventral region. Immunoreactivity to IGF-I could also be detected in the otic vesicle and the CVG. The mechanism of action of IGF was explored by studying the turnover of glycosylated phosphatidylinositols and Fos oncoprotein expression. IGF-I was able to rapidly increase Fos levels in cultured otic vesicles, and antisense oligonucleotides complementary to c-fos were able to inhibit IGF-I-induced growth. Both IGF-I-induced cell proliferation and Fos expression were blocked by treatment with an anti-inositol phosphoglycan antibody. It is proposed that IGF-I displays the requisites to be the factor which regulates growth of the otic primordium during normal development of the inner ear.

* This work was done in collaboration with F. Giraldez and J. Represa. Instituto de Biología y Genética Molecular (IBGM) - Depto. Anatomía, Dpto. Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, 47005 Valladolid, Spain.

CONCLUDING REMARKS

F. de Pablo

Since the advent of experimental embryology, about a century ago, until the last decade, very little progress was made in understanding the signals controlling embryonic induction, patterning and cell growth and differentiation in development. During the last decade, an explosion of studies have identified factors, some of them known previously for their role in classic endocrinology and immunology arenas, and many new gene products, that drive the developmental program of vertebrate organisms. The use of sophisticated cellular and molecular approaches (clonal cultures, *in situ* hybridization, PCR, gene targetted knockouts, confocal microscopy, etc.) permits now to reanalyze with better tools the old problem of how an egg becomes a fully developed organism.

This workshop was an excellent forum where 19 invited speakers and 29 participants discussed their progress towards elucidation of factors that influence the fate and survival of cells, and how they may interact in the combinatorial complexity of cellular signaling. Inductive, hemopoietic, differentiation, neurotrophic and proliferation/survival factors, were analyzed in a wide range of developmental stages, from gastrulation to perinatal, in human, rodent, chicken and amphibian models.

Special contribution were the short presentations of several excellent posters, chosen among the 23 brought by participants. These included the work on the zinc finger gene *slug* involved in the epithelial-mesenchymal transition (A. Nieto), the multispecies analysis of oligodendrocyte lineage, using the PDGF-αR as a marker (W.D. Richardson) and the disruption of axis formation in fish by ectopical expression of activin- variants (J. Wittbrodt).

From the meeting active interchange of ideas it has become clear that a large combination of factors acting in partially overlapping fashion (the concept of redundancy of genes was, by most scientist, found unacceptable!), are likely to be involved in the sophisticated orchestration of cell-to-cell signaling from early to late develoment. We still do not understand the mechanism by which pleiotropic cytokines act in some cellular settings as proliferative factors and in

others as differentiative factors or survival factors. A great deal will be learned from mice lacking one or various combinations of factors and receptors; even more informative will be null-mutations done in a tissue-specific, time-controlled fashion. The traditional transplantation experiments and organocultures, using the appropiate gene markers, have a lot to add as well to the field. We all hope that developmental biology continues to be the theme of successful meetings sponsored by the Fundación Juan March.

List of Invited Speakers

Workshop on

ROLES OF GROWTH AND CELL SURVIVAL FACTORS IN VERTEBRATE DEVELOPMENT

List of Invited Speakers

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List of Participants

Workshop on

ROLES OF GROWTH AND CELL SURVIVAL FACTORS
IN VERTEBRATE DEVELOPMENT

List of Participants

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Texts published in the
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concerning workshops and courses organized within the
Plan for International Meetings on Biology (1989-1991)

246 **Workshop on Tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening, J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on the Polymerase Chain Reaction.**
Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

266 **Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects.**
Organized by F. X. Avilés. Lectures by T. L. Blundell, W. Bode, P. Carbonero, R. W.Carrell, C. S. Craik, T. E. Creighton, E. W. Davie, L. D. Fricker, H. Fritz, R. Huber, J. Kenny, H. Neurath, A. Puigserver, C. A. Ryan, J. J. Sánchez-Serrano, S. Shaltiel, R. L. Stevens, K. Suzuki, V. Turk, J. Vendrell and K. Wüthrich.

267 **Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling.**
Organized by J. M. Mato and J. Larner. Lectures by M. V. Chao, R. V. Farese, J. E. Felíu, G. N. Gaulton, H. U. Häring, C. Jacquemin, J. Larner, M. G. Low, M. Martín Lomas, J. M. Mato, E. Rodríguez-Boulan, G. Romero, G. Rougon, A. R. Saltiel, P. Strålfors and I. Varela-Nieto.

268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
Organized by R. Serrano and J. A. Pintor-

Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

269 **Workshop on Neural Control of Movement in Vertebrates.**
Organized by R. Baker and J. M. Delgado-García. Lectures by C. Acuña, R. Baker, A. H. Bass, A. Berthoz, A. L. Bianchi, J. R. Bloedel, W. Buño, R. E. Burke, R. Caminiti, G. Cheron, J. M. Delgado-García, E. E. Fetz, R. Gallego, S. Grillner, D. Guitton, S. M. Highstein, F. Mora, F. J. Rubia Vila, Y. Shinoda, M. Steriade and P. L. Strick.

Texts published by the CENTRE FOR INTERNATIONAL MEETINGS ON BIOLOGY

1 **Workshop on What do Nociceptors Tell the Brain?**
Organized by C. Belmonte and F. Cerveró. Lectures by C. Belmonte, G. J. Bennet, J. N. Campbell, F. Cerveró, A. W. Duggan, J. Gallar, H. O. Handwerker, M. Koltzenburg, R. H. LaMotte, R. A. Meyer, J. Ochoa, E. R. Perl, H. P. Rang, P. W. Reeh, H. G. Schaible, R. F. Schmidt, J. Szolcsányi, E. Torebjörk and W. D. Willis Jr.

2 **Workshop on DNA Structure and Protein Recognition.**
Organized by A. Klug and J. A. Subirana. Lectures by F. Azorín, D. M. Crothers, R. E. Dickerson, M. D. Frank-Kamenetskii, C. W. Hilbers, R. Kaptein, D. Moras, D. Rhodes, W. Saenger, M. Salas, P. B. Sigler, L. Kohlstaedt, J. A. Subirana, D. Suck, A. Travers and J. C. Wang.

3 **Lecture Course on Palaeobiology: Preparing for the Twenty-First Century.**
Organized by F. Álvarez and S. Conway Morris. Lectures by F. Álvarez, S. Conway Morris, B. Runnegar, A. Seilacher and R. A. Spicer.

4 **Workshop on The Past and the Future of Zea Mays.**
Organized by B. Burr, L. Herrera-Estrella and P. Puigdoménech. Lectures by P. Arruda, J. L. Bennetzen, S. P. Briggs, B. Burr, J. Doebley, H. K. Dooner, M. Fromm, G. Gavazzi, C. Gigot, S. Hake, L. Herrera-Estrella, D. A. Hoisington, J. Kermicle, M. Motto, T. Nelson, G. Neuhaus, P. Puigdomenech, H. Saedler, V. Szabo and A. Viotti.

5 **Workshop on Structure of the Major Histocompatibility complex.**
Organized by A. Arnaiz-Villena and P. Parham. Lectures by A. Arnaiz-Villena, R. E. Bontrop, F. M. Brodsky, R. D. Campbell, E. J. Collins, P. Cresswell, M. Edidin, H. Erlich, L. Flaherty, F. Garrido, R. Germain, T. H. Hansen, G. J. Hämmerling, J. Klein, J. A. López de Castro, A. McMichael, P. Parham, P. Stastny, P. Travers and J. Trowsdale.

6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

7 **Workshop on Transcription Initiation in Prokaryotes.**
Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
Organized by C. Gancedo and J. M. Gancedo. Lectures by T. G. Cooper, T. F. Donahue, K.-D. Entian, J. M. Gancedo, C. P. Hollenberg, S. Holmberg, W. Hörz, M. Johnston, J. Mellor, F. Messenguy, F. Moreno, B. Piña, A. Sentenac, K. Struhl, G. Thireos and R. S. Zitomer.

10 **Workshop on Engineering Plants Against Pests and Pathogens.**
Organized by G. Bruening, F. García-Olmedo and F. Ponz. Lectures by R. N. Beachy, J. F. Bol, T. Boller, G. Bruening, P. Carbonero, J. Dangl, R. de Feyter, F. García-Olmedo, L. Herrera-Estrella, V. A. Hilder, J. M. Jaynes, F. Meins, F. Ponz, J. Ryals, J. Schell, J. van Rie, P. Zabel and M. Zaitlin.

11 **Lecture Course on Conservation and Use of Genetic Resources.**
Organized by N. Jouve and M. Pérez de la Vega. Lectures by R. P. Adams, R. W. Allard, T. Benítez, J. I. Cubero, J. T. Esquinas-Alcázar, G. Fedak, B. V. Ford-Lloyd, C. Gómez-Campo, V. H. Heywood, T. Hodgkin, L. Navarro, F. Orozco, M. Pérez de la Vega, C. O. Qualset, J. W. Snape and D. Zohary.

12 **Workshop on Reverse Genetics of Negative Stranded RNA Viruses.**
Organized by G. W. Wertz and J. A. Melero. Lectures by G. M. Air, L. A. Ball, G. G. Brownlee, R. Cattaneo, P. Collins, R. W. Compans, R. M. Elliott, H.-D. Klenk, D. Kolakofsky, J. A. Melero, J. Ortín, P. Palese, R. F. Pettersson, A. Portela, C. R. Pringle, J. K. Rose and G. W. Wertz.

13 **Workshop on Approaches to Plant Hormone Action.**
Organized by J. Carbonell and R. L. Jones. Lectures by J. P. Beltrán, A. B. Bleecker, J. Carbonell, R. Fischer, D. Grierson, T. Guilfoyle, A. Jones, R. L. Jones, M. Koornneef, J. Mundy, M. Pagès, R. S. Quatrano, J. I. Schroeder, A. Spena, D. Van Der Straeten and M. A. Venis.

14 **Workshop on Frontiers of Alzheimer Disease.**
Organized by B. Frangione and J. Ávila. Lectures by J. Ávila, K. Beyreuther, D. D. Cunningham, A. Delacourte, B. Frangione, C. Gajdusek, M. Goedert, Y. Ihara, K. Iqbal, K. S. Kosik, C. Milstein, D. L. Price, A. Probst, N. K. Robakis, A. D. Roses, S. S. Sisodia, C. J. Smith, W. G. Turnell and H. M. Wisniewski.

15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
Organized by J. M. Mato and A. Ullrich. Lectures by M. Barbacid, A. Bernstein, J. B. Bolen, J. Cooper, J. E. Dixon, H. U. Häring, C.- H. Heldin, H. R. Horvitz, T. Hunter, J. Martín-Pérez, D. Martín-Zanca, J. M. Mato, T. Pawson, A. Rodríguez-Tébar, J. Schlessinger, S. I. Taylor, A. Ullrich, M. D. Waterfield and M. F. White.

16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

17 **Workshop on Cell Recognition During Neuronal Development.**
Organized by C. S. Goodman and F. Jiménez. Lectures by J. Bolz, P. Bovolenta, H. Fujisawa, C. S. Goodman, M. E. Hatten, E. Hedgecock, F. Jiménez, H. Keshishian, J. Y. Kuwada, L. Landmesser, D. D. M. O'Leary, D. Pulido, J. Raper, L. F. Reichardt, M. Schachner, M. E. Schwab, C. J. Shatz, M. Tessier-Lavigne, F. S. Walsh and J. Walter.

18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
Organized by C. Nathan and A. Celada. Lectures by D. O. Adams, M. Aguet, C. Bogdan, A. Celada, J. R. David, A. Ding, R. M. Fauve, D. Gemsa, S. Gordon, J. A. M. Langermans, B. Mach, R. Maki, J. Mauël, C. Nathan, M. Röllinghoff, F. Sánchez-Madrid, C. Schindler, A. Sher and Q.-w. Xie.

19 Workshop on Viral Evasion of Host Defense Mechanisms.
Organized by M. B. Mathews and M. Esteban. Lectures by E. Domingo, L. Enjuanes, M. Esteban, B. N. Fields, B. Fleckenstein, L. Gooding, M. S. Horwitz, A. G. Hovanes-sian, M. G. Katze, I. M. Kerr, E. Kieff, M. B. Mathews, G. McFadden, B. Moss, J. Pavlovic. P. M. Pitha, J. F. Rodríguez, R. P. Ricciardi, R. H. Silverman, J. J. Skehel, G. L. Smith, P. Staeheli, A. J. van der Eb, S. Wain-Hobson, B. R. G. Williams and W. Wold.

20 Workshop on Genomic Fingerprinting.
Organized by McClelland and X. Estivill. Lectures by G. Baranton, D. E. Berg, X. Estivill, J. L. Guénet, K. Hayashi, H. S. Kwan, P. Liang, M. McClelland, J. H. Nadeau, D. L. Nelson, M. Perucho, W. Powell, J. A. Rafalski, O. A. Ryder, R. Sederoff, A. J. G. Simpson, J. Welsh and H. Zischler.

21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

22 Workshop on Molecular Bases of Ion Channel Function.
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

23 Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.
Organized by C. M. Thomas, E. M. H. Wellington, M. Espinosa and R. Díaz Orejas. Lectures by J. C. Alonso, F. Baquero, P. A. Battaglia, P. Courvalin, F. de la Cruz, D. K. Chattoraj, E. Díaz, R. Díaz Orejas, M. Espinosa, J. C. Fry, K. Gerdes, D. R. Helinski, B. Hohn, E. Lanka, V. de Lorenzo, S. Molin, K. Nordström, R. W. Pickup, C. M. Thomas, J. D. van Elsas, E. M. H. Wellington and B. Wilkins.

24 Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.
Organized by P. D. Coleman, F. Mora and M. Nieto-Sampedro. Lectures by J. Avila, Y.-A. Barde, P. D. Coleman, C. W. Cotman, Y. Lamour, M. P. Mattson, A. Matus, E. G. McGeer, B. S. Meldrum, J. Miquel, F. Mora, M. Nieto-Sampedro, V. H. Perry, M. P. Rathbone, G. S. Roth, R. R. Sturrock, H. B. M. Uylings and M. J. West.

25 Workshop on Genetic Recombination and Defective Interfering Particles in RNA Viruses.
Organized by J. J. Bujarski, S. Schlesinger and J. Romero. Lectures by V. Agol, P. Ahlquist, J. J. Bujarski, J. Burgyán, E. Domingo, L. Enjuanes, S. P. Goff, T. C. Hall, A. S. Huang, K. Kirkegaard, M. M. C. Lai, T. J. Morris, R. F. Ramig, D. J. Robinson, J. Romero, L. Roux, S. Schlesinger, A. E. Simon, W. J. M. Spaan, E. G. Strauss and S. Wain-Hobson.

26 Workshop on Cellular Interactions in the Early Development of the Nervous System of Drosophila.
Organized by J. Modolell and P. Simpson. Lectures by S. Artavanis-Tsakonas, J. A. Campos-Ortega, S. B. Carroll, C. Dambly-Chaudière, C. Q. Doe, R. G. Fehon, M. Freeman, A. Ghysen, V. Hartenstein, D. Hartley, Y. N. Jan, C. Klämbt, E. Knust, A. Martínez-Arias, M. Mlodzik, J. Modolell, M. A. T. Muskavitch, B.- Z. Shilo, P. Simpson, G. M. Technau, D. van Vactor and C. Zuker.

27 Workshop on Ras, Differentiation and Development
Organized by J. Downward, E. Santos and D. Martín-Zanca. Lectures by A. Balmain, G. Bollag, J. Downward, J. Erickson, L. A. Feig, F. Giráldez, F. Karim, K. Kornfeld, J. C. Lacal, A. Levitzki, D. R. Lowy, C. J. Marshall, J. Martín-Pérez, D. Martín-Zanca, J. Moscat, A. Pellicer, M. Perucho, E. Santos, J. Settleman, E. J. Taparowsky and M. Yamamoto.

28 Human and Experimental Skin Carcinogenesis
Organized by A. J. P. Klein-Szanto and M. Quintanilla. Lectures by A. Balmain, F. F.

Becker, J. L. Bos, G. T. Bowden, D. E. Brash, A. Cano, C. J. Conti, G. P. Dotto, N. E. Fusenig, J. L. Jorcano, A. J. P. Klein-Szanto, E. B. Lane, H. Nakazawa, G. Orth, M. Quintanilla, D. R. Roop, J. Schweizer, T. J. Slaga and S. H. Yuspa.

29 **Workshop on the Biochemistry and Regulation of Programmed Cell Death.**
Organized by J. A. Cidlowski, H. R. Horvitz, A. López-Rivas and C. Martínez-A. Lectures by J. C. Ameisen, R. Buttyan, J. A. Cidlowski, J. J. Cohen, M. K. L. Collins, T. G. Cotter, A. Eastman, D. R. Green, E. A. Harrington, T. Honjo, H. R. Horvitz, J. T. Isaacs, M. D. Jacobson, P. H. Krammer, A. López-Rivas, C. Martínez-A. S. Nagata, G. Núñez, R. W. Oppenheim, S. Orrenius, J. J. T. Owen, M. C. Raff, L. M. Schwartz and J. Yuan.

30 **Workshop on Resistance to Viral Infection.**
Organized by L. Enjuanes and M. M. C. Lai. Lectures by M. Ackermann, R. Blasco, L. Enjuanes, M. Esteban, P. A. Furth, F. L. Graham, L. Hennighausen, G. Keil, M. M. C. Lai, S. Makino, J. A. Melero, K. Olson, J. Ortín, E. Paoletti, C. M. Rice, H. L. Robinson, J. F. Rodríguez, B. Roizman, J. J. Rossi, P. Roy, S. G. Siddell, W. J. M. Spaan, P. Staeheli and M. Del Val.

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