Instituto Juan March
de Estudios e Investigaciones

40 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# Nuclear Oncogenes and Transcription Factors in Hematopoietic Cells

Organized by

J. León and R. Eisenman

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A. Berns
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*The lectures summarized in this publication were presented by their authors at a workshop held on the 24th through the 26th of April, 1995, at the Instituto Juan March.*

Depósito legal: M-19.397/1995
ISBN: 84-7919-540-1
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

# INDEX

# INTRODUCTION

Javier León and Robert Eisenman

## INTRODUCTION

**Robert Eisenman and Javier León**

Elucidation of the molecular mechanism underlying hematopoiesis poses both a challenge and an opportunity. All blood cells are derived from a common pool of stem cells which in turn generate a hierarchy of hematopoietic progenitors that undergo proliferation and differentiation along distinct cell lineages. At least nine mature cell types, differing greatly in morphology, function and population size, are generated from such hematopoietic stem cells. The problems associated with understanding the regulation of such a complex system are mind boggling. However leukemias have also provided us with hematopoietic cells in which normal regulation has been subverted and have thus permitted the identification of crucial control elements. Most of the genes involved in the control of proliferation and differentiation of hematopoietic cells were originally isolated because they were found mutated or rearranged in human leukemia or as oncogenes transduced by leukemogenic retroviruses.

Not surprinsingly, most of these leukemia-related oncogenes were found to encode transcription factors. Therefore these genes are thought to be "master genes" controlling the switch between quiescence and proliferation, between different cell lineages and programmed cell death. New advances in the structure, expression, and function of the proteins encoded by these genes have been disclosed in this workshop, held under the auspices of Instituto Juan March in Madrid.

During the workshop leading specialists presented new data on a number of transcription factors and their function in relation to cell proliferation, differentiation, apoptosis and malignant transformation. The factors covered in the workshop included Bcl6, Bmi, Fli1, Fos, ErbA, Ets1/2, Hlx, Hox11, IRF1/2, Jun, Mad, Max, Myc, Myb, p53, Pim1, Pml-RARα, Rbtn2, Spi1-PU1. For some of these genes data was presented on *in vivo* function, showing the alterations carried by transgenic animals or the phenotype produced by their targeted mutation in mice. For other genes, their involvement in differentiation or cell death was studied by using cell lines or expression pattern in animal tissues. Alterations of the genes found in leukemias and other malignancies were also described. The conference thus summarized the "state of the art" in terms of our knowledge of the biological roles of these hematopoietic regulatory genes and in the methodologies employed to augment our understanding.

We want to thank the invited speakers and the participants for their excellent contributions. Finally, we are deeply grateful to Andrés Gonzalez and the Instituto Juan March for the organization of this workshop.

# FIRST SESSION:
# TRANSCRIPTIONAL CONTROL OF HEMATOPOIESIS

Chairperson: Robert Eisenman

# LINEAGE COMMITMENT IN THE HEMATOPOIETIC SYSTEM

Jerry Adams, Geoffrey Lindeman, Alan Harris, Jane Visvader, Bernd Hentsch, Richard Harvey and Suzanne Cory - The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

A central, poorly understood issue in hematopoiesis is the nature of the network of transcription factors that presumably control lineage commitment, *i.e.* the process whereby the eight or more lineages of blood cells are derived continuously from a single pleuripotent stem cell. In addition to studies (not addressed here) on the role of GATA factor genes in megakaryocytic differentiation (e.g.1), our laboratory has been exploring the potential role of homeobox genes in hematopoiesis (2-4) as well as in leukemogenesis (5,6) Moreover, by serendipity, we have generated a mouse strain in which hematopoiesis is grossly disturbed (7).

Our recent homeobox studies have concentrated on the role of the *Hlx* gene (2-4). During embryonic development, *Hlx* is first expressed in visceral mesoderm and later in two hematopoietic lineages, myelomonocytic cells and B lymphoid cells. To determine its biological functions, we have disrupted the gene in embryonic stem cells and studied mice carrying the mutant allele. Homozygous embryos die around 15 days of development with anemia and a severe defect in liver development. Blood development is poor and exhibits a block in the switch to fetal hematopoiesis. Whether the hematopoietic defect is intrinsic to $Hlx^{-/-}$ cells or is an indirect consequence of the liver lesion is under study.

During microinjection of oocytes we have inadvertantly produced a novel mouse strain (denoted *max* 41) with disturbed hematopoiesis (7). These mice exhibit a gross excess of granulocytes and a concomitant dearth of B lymphocytes. The perturbation appears to be cell autonomous and several observations suggest that B lymphopoiesis in these mice has been diverted to granulocyte production. We hypothesize that integration of the transgene has inadvertantly altered expression of an endogenous locus which can determine lineage commitment. These findings suggest unexpected plasticity in hematopoietic programming.

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# Mouse Mutants Carrying Gain or Loss of Function Mutations in the *Fli* Transcription Factor Gene

**Alan Bernstein**
Div. of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, Canada.

The *ets* genes constitute a large and evolutionary conserved family of genes that code for transcription factors involved in development and malignant transformation. The founding member of the *ets* family, the oncogene v-*ets*, was originally discovered in 1983 as part of a fusion protein with *gag* and *myb*, in the genome of E26, an avian erythroblastosis virus. Since that time, novel *ets* genes have been discovered by sequence homology, by genetic screens in the fly, by cloning the common sites of proviral integration in retrovirally-induced leukemias, by cloning the genes that encode proteins that bind to sequence-specific DNA elements, and by cloning the sites of chromosomal translocations in human malignancies.

Our laboratory has been interested in the *Fli*-1 *ets* gene, discovered by us as a common Friend leukemia virus integration site in the erythroleukemias induced by this virus. Subsequently, *Fli*-1 was shown to be rearranged in Ewings sarcoma and neuroepithelioma, two common pediatric tumors, as a result of a reciprocal t(11;22) chromosomal translocation.

In order to understand the role of *Fli*-1 in normal cells, we have generated both transgenic mice in which the *Fli*-1 coding region is driven by the H-$2^K$ promoter and targeted mice in which a null mutation in the *Fli*-1 gene has been introduced into the mouse germ-line by gene targeting in ES cells. Phenotypic analysis of gain and loss of function mutations in the *Fli*-1 should provide insights into the normal role of the *Fli*-1 transcription factor in development and in the adult animal.

Transgenic mice that over-express *Fli*-1 develop at high incidence a progressive immunological renal disease and ultimately die of renal failure due to immune-complex glomerulonephritis. These data, together with the observation that *Fli*-1 is most highly expressed in the thymus, suggests that even a modest elevation in *Fli*-1 expression can perturb normal T cell development, leading to a severe autoimmune disorder.

To address these issues further, we generated a null mutation in the *Fli*-1 gene by gene targeting in ES cells. *Fli*-1 heterozygous mice are apparently normal, suggesting that a 50% reduction in the levels of *Fli*-1 protein has no discernible effect. Matings between heterozygous *Fli*-1 mice yielded progeny of all three genotypes, including the predicted number of mice *Fli*-$1^{(-/-)}$ homozygous mice. We conclude that *Fli*-1 is not essential for embryonic development or viability, perhaps because other members of the *ets* gene family, particularly *erg* which is very close in sequence to *Fli*-1, functionally replaces the *Fli*-1 protein in these mice. Because *Fli*-1 is highly expressed in the thymus and many *ets* genes, including *Fli*-1, recognize and bind to specific sequences in the promoter regions of a number of lymphoid-specific genes, we have begun a more detailed examination of T cell development in *Fli*-$1^{(-/-)}$ mice. Preliminary observations suggest that there are, indeed, alterations in thymocyte development in the *Fli*-$1^{(-/-)}$ mice, the nature of which is currently under investigation.

# Role of v-Myb in differentiation, growth control and survival of haematopoietic cells

**Jon Frampton and Thomas Graf.** Differentiation Programme, European Molecular Biology Laboratory, Meyerhofstraße 1, 69117 Heidelberg, Germany.

The E26 acute avian leukaemia virus encodes a fused protein derived from truncated versions of the Myb and Ets-1 transcription factors. Infection of haematopoietic cells *in vitro* induces the clonal expansion of two types of haematopoietic cells; myeloblasts and multipotent progenitors (MEPs). The myeloblasts require cMGF for growth and exhibit immature granulocyte/macrophage markers. MEPs have phenotypic characteristics of thromboblasts but can differentiate spontaneously into erythroid cells and can be induced to differentiate into either myeloblasts or eosinophils with phorbol ester.

In earlier work, a mutant in E26 v-Myb (*ts*21) was isolated that transforms myeloblasts at 37°C but not at 42°C. This results from loss of the capacity of the mutated v-Myb component to bind to DNA. *Ts*21 transformed myeloblasts shifted to 42°C differentiate into macrophages, while *ts*21 transformed MEPs differentiate into cells resembling mature thrombocytes which express a number of thrombocyte specific surface and cytoplasmic markers and have the ability to store and release serotonin. These results indicate that v-Myb plays a role in blocking the differentiation of both myelomonocytic and thrombocytic progenitors; this is quite distinct from the lineages affected by the Ets portion of the fusion protein.

Recently, we have found that in transformed myeloblasts, but not in MEPs, v-Myb inactivation additionally leads to the rapid death of cells by apoptosis. In contrast, withdrawal of the myelomonocytic growth factor cMGF causes a block to proliferation and eventual apoptosis over a period of > 5 days. The apoptosis induced by Myb inactivation correlated with a dramatic drop in *bcl*-2 RNA levels at 42°C, suggesting that v-Myb controls the expression of the *bcl*-2 gene and that this might explain its influence on apoptosis. The observation that *bcl*-2 RNA reappears within 2 hours upon reactivation of v-Myb by shift back from 42°C to 37°C suggests that it is directly regulated by v-Myb. In support of this, the E26 fusion protein acts as a strong transactivator of the *bcl*-2 promoter in transient transfection assays. Apoptosis could be prevented in *ts*21 transformed myeloblasts at 42°C by over-expression of *bcl*-2.

Our data suggest that myeloid cells require at least two independent signals for their survival; that is, Myb and growth factor. In addition, the up-regulation of *bcl*-2 expression explains, at least in part, the myeloid cell transforming capacity of v-Myb.

# *Designed DNA-binding proteins as specific inhibitors of transcription*

YEN CHOO

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Site-specific DNA-binding proteins designed to recognise predetermined sequences could exploit genetic differences in pathogens or transformed cells as direct targets for therapeutic agents in the treatment of disease.

Zinc fingers capable of recognising given DNA triplets can be isolated by affinity selections from libraries of zinc finger peptides displayed on filamentous phage; and the specificity of these fingers can be reliably checked in a single-step by screening against libraries of positionally randomised DNA oligonucleotides. Since zinc fingers are modular DNA-binding domains, sequence specific DNA-binding proteins can be built by the appropriate combination of selected fingers in a covalent concatemer. Designed proteins containing three fingers can bind to 9bp asymmetric sites with $K_d$s in the nanomolar range, and to sites differing by 1bp with $K_d$s higher usually by at least a factor of ten.

Such designed DNA-binding proteins could be used to regulate the expression of a given gene at the level of transcription, before the amplification of genetic information to RNA or protein. Conventionally this could be accomplished by the fusion of the DNA-binding domain to transcriptional activation or silencing domains which influence the rate of initiation of RNA synthesis. Alternatively, the DNA-binding domain alone bound to the coding sequence of particular genes may be able to specifically arrest (but not stimulate) transcription by blocking the path of RNA polymerase.

To date, the principle of the latter strategy has been demonstrated by the engineering of a zinc finger peptide able to bind specifically to a unique region of a *BCR-ABL* oncogene in preference to the parent genomic sequences. Using transformed cells in culture as a model, it has been shown that binding to the target site in chromosomal DNA is possible, and results in blockage of transcription. Consequently, transformed cells rendered growth-factor independent by the action of the oncogene are found to revert to factor dependence on transient transfection with a vector expressing the peptide.

Analysis of the synergism of c-Myb and NF-M on the mim-1 promoter
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Institute of Molecular Biology, Hannover Medical School, Konstanty-Gutschow-Strasse 8, 30623 Hannover, Germany

c-Myb is a transcriptional activator that plays an essential role in the regulation of hematopoiesis. mim-1 the first gene identified to be regulated by c-Myb *in vivo* contains three Myb binding sites in its promoter region. The most proximal one (MRE A) is sufficient to render the promoter c-Myb responsive in transient transfection experiments. The MRE A is flanked by a C/EBP binding site and cotransfection of c-Myb and NF-M, the chicken homologue of the mammalian C/EBPβ, results in a synergistic activation of the mim-1 promoter. We were interested to understand the mechanism of this synergism. In the first series of experiments electrophoretic mobility shift assays were performed to test if c-Myb and NF-M could cooperate in DNA binding using an oligonucleotide derived from the mim-1 promoter that contained the MRE A and the C/EBP site. No cooperative binding of c-Myb and NF-M to this oligonucleotide could be detected. Therefore it seems unlikely that the synergism is conferred by direct interaction or by facilitated binding of these two factors to the mim-1 promoter. Another possibility is that a third factor has to be recruited by both c-Myb and NF-M for efficient transactivation. Recently we identified a protein that can interact with c-Myb *in vitro* and mapped the interaction domains. Co-expression of the protein in transient transfections stimulated dose dependently the c-Myb specific transactivation of a mim-1 reporter gene in various cell lines. In contrast this factor did not stimulate NF-M dependent mim-1 transcription. However NF-M transactivation activity was stimulated by cotransfected Ha-Ras and after its activation became responsive to our factor. At present we are investigating if NF-M can interact with our protein directly. Since c-Myb and NF-M are both stimulated in their transactivation activity by our factor we propose that this protein can play an important role in mediating their synergistic activation of the mim-1 promoter.

# Rapid downregulation of thrombospondin mRNA in avian cells infected by a v-Myc retrovirus

**Andrei T.Tikhonenko**, Daniel J.Black, and Maxine L.Linial
Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104, U.S.A.

We are interested in identifying the transcriptional targets of the retroviral v-Myc oncoprotein. To this end, we have engineered a conditional mutant of v-Myc, designated GRIM, bearing the ligand binding domain of the rat glucocorticoid receptor. It thus requires the presence of hormone to induce neoplastic transformation. We introduced the wild type GRIM retrovirus (GRIM*wild*) along with its transformation-defective derivative GRIM*lost* into avian embryo fibroblasts and maintained both cultures (GW and GL) in hormone-free medium. Dexamethasone, a synthetic ligand for GR, was then added to mobilize v-Myc and thus allow transcription of its putative targets. Four hours post stimulation, mRNAs were extracted from GW- and GL-cells and the corresponding cDNA libraries were constructed. Replica filters of these libraries were analyzed using GW- and GL-specific radiolabelled RNAs as the molecular probes. Hybridization screening yielded one clone encoding mRNA underrepresented in GW-library. Consistently, the corresponding mRNA was expressed in GRIM*wild*-cells at much lower levels than in control GRIM*lost*-infected cells.

Sequence analysis revealed that this mRNA was virtually identical to the 3' untranslated region of the human thrombospondin 1 mRNA encoding a secreted glycoprotein. Since thrombospondin expression is often repressed in transformed cells, we wanted to confirm that in this instance it has happened in immediate response to v-Myc activation. Indeed, we were able to demonstrate that in GRIM-infected cells thrombospondin downregulation occurred even in the absence of *de novo* protein synthesis and was apparent as early as 5 hours after addition of dexamethasone. We are currently examining what effect forced re-expression of thrombospondin might have on transformation by v-Myc.

Control of cell proliferation by *c-myc*

Philipp Steiner , Bettina Rudolph and Martin Eilers, Zentrum für Molekulare Biologie Heidelberg (ZMBH); Im Neuenheimer Feld 282, 69120 Heidelberg , FRG

The product of the proto-oncogene c-Myc is one of the key regulators of mammalian cell proliferation. Myc protein is a transcription factor of the HLH/LZ family. Although several genes are known that are regulated by Myc, the mechanism by which Myc triggers cell cycle progression remains unclear. In particular none of the cyclin/cdk genes expressed early in the cell cycle is strongly induced by Myc. We now report that induction of Myc in density-arrested fibroblasts triggers rapid hyperphosphorylation of the retinoblastoma protein and activation of both cyclin D1 and cyclin E associated kinase activities in the absence of significant changes in the amounts of cyclin/cdk complexes. Kinase activation by Myc is blocked by inhibitors of transcription and requires intact DNA binding and heterodimerization domains of Myc. Dominant negative alleles of cdk2, antibodies against cyclin D1 and inhibitors of cyclin-dependent kinases all block cell cycle induction by Myc, demonstrating that activation of both D1/cdk4 and E/cdk2 kinases is required for cell cycle regulation by Myc. In serum-starved cells, activation of cyclin E/cdk2 kinase occurs in two steps: the first occurs in response to Myc and involves the release of a 120 kD cyclin E/cdk2 complex from a 250 kD inactive complex that is present in starved cells. This is necessary, but not sufficient to generate full kinase activity as cdc25 phosphatase activity is limiting in the absence of external growth factors. In vivo, cdc25 activity can be supplied by the addition of growth factors. In vitro, recombinant cdc25a strongly activates the 120 kD, but only poorly activates the 250 kD cyclin E/cdk2 complex. Our data show that two distinct signals, one of which supplied by Myc, are necessary for consecutive steps during growth factor-induced formation of active cyclin E/cdk2 complexes in Go-arrested rodent fibroblasts.

# SECOND SESSION:
# TRANSCRIPTIONAL MECHANISMS UNDERLYING HEMATOPOIETIC NEOPLASIA

Chairperson: Gerard Evan

**Molecular genetics of Acute Promyelocytic Leukemia (APL).** Myriam Alcalay, Francesco Grignani, Marta Fagioli, Pier Francesco Ferrucci, Daniela Rogaia, Lucia Tomassoni, Martin Ruthardt and Pier Giuseppe Pelicci. Istituto di Medicina Interna e Scienze Oncologiche, Policlinico Monteluce, Perugia University, 06100 Perugia, Italy

The t(15;17) is detected in 70-80% of APLs. The translocation breakpoints are located within the gene that encodes the retinoic acid receptor alpha (*RARa*) on 17 and a previously unknown gene, *PML*, on 15. The t(15;17) is a balanced reciprocal translocation that leads to the formation of two functional chimeric genes: *PML\RARa* and the reciprocal *RARa\PML*. The candidate transforming gene is *PML\RARa*, since it is expressed in 100% cases, whereas *RARa\PML* is expressed in only 70-80%. We have recently identified APL cases with submicroscopic 15;17 recombinations leading to the formation of non-reciprocal *PML\RARa* or *RARa\PML* fusion genes. Each of the two reciprocal translocation products may, therefore, be independently formed and selected by the leukemic phenotype, implying that both are involved in tumorigenesis. Retinoic Acid (RA) treatment induces disease remission in most cases. **Effects of PML/RARa on differentiation and survival.** We expressed the PML/RARa protein in the U937 myeloid precursor cells and showed that they: i) lose the capacity to differentiate when induced by vitamin $D_3$ and TGFb1; ii) acquire enhanced sensitivity to the differentiation action of RA; iii) exhibit a higher growth rate that is accounted for by a reduction in apoptotic cell death. These biological activities of PML/RARa recapitulate critical features of the APL phenotype, e.g. block of differentiation and high sensitivity to RA differentiation. To more deeply investigate the effects of PML/RARa on survival we expressed the fusion protein in the growth-factor dependent TF-1 cell line. PML/RARa expression markedly delayed apoptotic cell death without inducing growth factor independence. Growth factor deprivation caused rapid and massive apoptosis of control TF-1 cells. Factor-deprived cells were rapidly, synchronously and irreversibly committed to apoptosis as shown by their inability to re-enter the cell cycle after GM-CSF treatment. The percentage of PML/RARa expressing apoptotic cells was low but constant over the four weeks of factor deprivation. GM-CSF stimulated proliferation of factor-deprived cells at all time points during the 4 weeks of culture, suggesting that commitment to apoptosis was asynchronous and delayed in PML/RARa expressing TF-1 cells. We conclude that PML/RARa interferes with the genetic pathways of survival by reducing the frequency of commitment to apoptosis. **Mechanisms of PML/RARa action.** PML/RARa heterodimerizes with PML and the nuclear localization of the heterodimer in APL cells differs from that of PML homodimers. PML/RARa has micropunctated nuclear pattern, PML has a speckled nuclear pattern, whereas RARa is finely dispersed in the nucleus. Anti-PML antibodies reveal that APL cells display the PML/RARa-like micropunctated nuclear pattern, indicating that the fusion protein localization dominates over the two wild type proteins. Strikingly, the treatment of APL blasts with RA converts the micropunctated nuclear pattern to the speckled, PML-like, pattern. PML/RARa also forms homodimers which bind to RAREs with a different specificity than RAR/RXR heterodimers. Finally, PML/RARa forms heterodimers with RXR. Formation of PML/RARa-RXR heterodimers can indirectly influence the functional activity of other nuclear receptors like RARs, TR and DR, which physiologically dimerize with RXR allowing efficient formation of stable RE-bound complexes. We have expressed a number of mutant PML/RARa molecules into U937 and TF-1 cells to dissect the contribution of different portions of the fusion protein to its biological activities.

**IDENTIFICATION AND CHARACTERIZATION OF SYNERGIZING ONCOGENES IN LYMPHOMAGENESIS.**

A. Berns. Division of Molecular Genetics of the Netherlands Cancer Institute, Amsterdam.

Transgenic mice overexpressing a number of oncogenes in their lymphoid compartment are predisposed to lymphomagenesis. They develop monoclonal tumors after a variable latency period, indicating that tumorigenesis in these mice requires additional stochastic events. To define these additional events, lymphomagenesis was accelerated by infecting mice overexpressing various oncogenes in their lymphoid compartment with a non-acute transforming murine leukemia virus. These viruses can activate collaborating proto-oncogenes by insertional mutagenesis while at the same time marking these genes thereby facilitating their molecular cloning. We expected that proviral tagging in transgenic Eμ-*myc* mice would uncover genes that efficiently synergizes with *myc* in transformation. This appeared to be the case. A number of common insertion sites were found that carry genes collaborating with *myc* : *pim-1, pim-2, bmi-1*, and *pal-1*. MuLV infection of Eμ-*pim*-1 transgenic mice showed *c-myc, N-myc, pal-1* and *tiam-1* as frequent collaborators. In some of the lymphomas more than one oncogene appeared to be activated by proviral insertion indicating that proviral insertional mutagenesis can mediate the genetic changes required for consecutive steps in tumorigenesis. Some of these genes are never found activated simultaneously within the same tumor clone, suggesting that they act on the same or similar downstream target and therefore belong to the same complementation group in transformation. Interestingly, proviral insertional mutagenesis could also be exploited to mark genes involved in later stages of the tumorigenic process. Upon transplanting primary tumors into syngeneic hosts it was possible to identify provirally activated genes that contributed to later stages of the tumorigenic process. Cells carrying integrations near these genes grew out preferentially upon transplantation. One of the genes identified in this fashion is *frat-1*. Some of the biological characteristics that can be mediated by *frat-1* will be discussed.

To expand our understanding of the transforming potential as well as the normal function of these newly identified genes we have generated mice in which these genes were these genes were disrupted by homologous recombination in embryonic stem cells (*pim-1, pim-2. bmi-1, frat-1).* Loss of function mutations in *pim-1* gave rise to a subtle phenotype whereas loss-of-*bmi-1* showed a profound effect. The latter mice were smaller, showed neurological abnormalities and a strongly impaired lymphopoiesis. Interestingly, also developmental defects were noted such as posterior transformation of the axial skeleton, an effect which is in line with *bmi-1* being a member of the *polycomb* group of genes that are involved in the maintenance of homeobox gene suppression. Indeed loss-of-function of *bmi-1* caused a shift in the boundary of expression of

some of the homeobox genes. In the lymphoid compartment aberrant expression of *bmi-1* appeared to affect a specific subset of B and T lymphocytes in line with its oncogenic potential in this tissue.

To gain further insight into the pathways in which these genes act in tumorigenesis we have introduced the Eμ-*myc* transgene into the *pim-1*$^{-/-}$ mutant background and induced tumors by MuLV infection. The rationale was that in view of the strong collaborative effect between myc and Pim-1 we might specifically target genes that could substitute for *pim-1* in transformation. Our data show that this approach can indeed give access to genes that fall into the same complementation group of transformation as *pim-1*. This validates this apporach of "suppression tagging" to identify key components in specific signal transduction pathways.

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**TRANSCRIPTIONAL PROPERTIES OF SUBGROUPS OF LYMPHOMA-DERIVED C-MYC MUTANTS: ENHANCED TRANSCRIPTIONAL SUPPRESSION OR DEFECTIVE REGULATION BY THE GROWTH SUPPRESSOR P107.** C.V. Dang, A.T. Hoang, L.A. Lee, B.C. Lewis, B. Lutterbach, T. Yano, M. Raffeld, S.R. Hann. Division of Hematology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD, Department of Cell Biology, Vanderbilt University, Nashville, TN, and Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD.

In addition to the characteristic chromosomal translocations involving the MYC gene in Burkitt and AIDS-related lymphomas, missense mutations are frequently found in MYC exon 2. We sought to determine the biological significance of these mutations and delineate the molecular properties of these mutant Myc proteins. We have found that a group of lymphoma-derived mutant MYC alleles, which encode missense mutations in the Myc transactivation domain, are more potent than wild-type MYC in transforming fibroblasts. p107, a Rb-related protein, suppresses transactivation by wild-type but not mutant chimeric GAL4-Myc activators. The mutations, however, do not disrupt the binding of Myc to p107 *in vitro* or *in vivo*. Mutation of Myc Thr-58, an *in vivo* phosphorylation site frequently altered in lymphomas and in retroviral v-Myc, results in increased transforming activity and causes resistance to suppression of transactivation by p107. Many lymphoma-derived Myc mutants have mutations flanking Thr-58 that may alter the consensus motif for kinases. In one such Burkitt lymphoma cell line, B3CL, *in vivo* phosphorylation of the mutant Myc Thr-58 is absent. *In vitro* studies show further that a p107-cyclin A-CDK complex is able to phosphorylate the N-terminal region of c-Myc at Ser-62. Other sites in the Myc transactivation domain, Thr-58 and Ser-71, are also phosphorylated *in vitro* in a fashion dependent on cyclin A. Two representative Myc mutants remained unphosphorylated at Thr-58 under identical conditions *in vitro*. We also studied the mutation affecting Phe-115 that lies within a Myc domain required for transcriptional suppression through the initiator element (Inr). Mutation of Phe-115 increases the transforming activity of c-Myc and the potential to suppress transcription through the Inr. These observations suggest that mutations of MYC disrupting a functional interaction with p107 and altering phosphorylation of c-Myc Thr-58 may be important in the genesis of some lymphomas with 8q24 translocations. Mutations affecting Phe-115 do not disrupt an interaction with p107, but appear to augment the ability of Myc to suppress transcription through the Inr. These observations suggest that somatic mutations in the c-Myc transactivation domain may sugment c-Myc transforming activity by altering its transcriptional properties.

BCL-6, A ZINC-FINGER TRANSCRIPTION FACTOR INVOLVED IN B CELL DIFFERENTIATION AND LYMPHOMAGENESIS. R. Dalla-Favera, Departments of Pathology and Genetics & Development, Columbia University, New York, NY 10032.

A substantial fraction (30-40%) of diffuse large-cell lymphoma (DLCL), the most frequent and clinically relevant type of non-Hodgkin lymphoma (NHL), is associated with rearrangements of the BCL-6 gene (1-3). These lesions have clinical significance since they identify a subset of DLCL characterized by extranodal origin and favorable prognosis (4).

The BCL-6 gene encodes a 95 kD nuclear phosphoprotein containing six C-terminal $C_2H_2$-type zinc-fingers and a N-terminal ZIN/POZ domain, a putative protein-protein interaction domain common to a subset of zinc-finger proteins including several Drosophila developmental regulators (2). BCL-6 binds to a specific DNA sequence through its zinc fingers and contains a potent transcriptional repressor domain. BCL-6 expression is regulated during B cell differentiation, being progressively upregulated from pre-B to mature B cells and then down regulated in terminally differentiated plasmacells. In lymphoid organs, the BCL-6 protein can be found in mature B cells within germinal centers, suggesting a role in determining the fate of B cells to become plasmacells, memory cells or die by apoptosis.

As a consequence of rearrangements affecting band 3q27, the BCL-6 promoter region is truncated and the coding domain is linked downstream to heterologous promoters translocated from other chromosomes. In addition to chromosomal rearrangements, a recent study revealed the presence of somatic mutations within a 4 kb region spanning the BCL-6 first non-coding exon in 14 of 18 DLCL cases (78%) carrying unrearranged BCL-6 genes, but not in 190 other biopsies including most types of solid tumors. Thus, considering both rearrangements and mutations, >80% of DLCL carry alterations in the 5' non coding region of BCL-6 . The frequency, clustering, and disease-association of these alterations suggest that they may contribute to lymphomagenesis, presumably by altering BCL-6 gene expression.

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## Oncogenic transcription factors activated by chromosomal translocations in T cell acute leukaemia: RBTN2 and HOX11.

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Leukaemias are characterised by the presence of chromosomal abnormalities, in particular chromosomal translocations and inversions. T cell acute leukaemias are characterised by a number of different recurring chromosomal translocations even though the disease in clinically homogeneous. The breakpoints of a number of these translocations have been cloned and the relevant genes isolated. A number of common features apply to the genes activated by T cell acute leukaemia chromosomal translocations. Firstly the genes are usually not normally expressed in T cells, secondly these are known or putative transcription factors and thirdly they are developmentally regulated genes. Examples of these are the RBTN2 and HOX11 genes, which are activated by distinct chromosomal translocations. The features of these genes, their expression, their role in tumorigenesis and their normal function is characteristic of this group of oncogenes and will be discussed.

*RBTN2* is located on chromosome 11, short arm band p13, at the junction of the translocation t(11;14)(p13;q11) which involves the T cell receptor $\alpha$ or $\delta$ genes from chromosome 14q11. More rarely a variant form of this translocation is found t(7;11)(q35;p3) which involves the T cell receptor $\beta$ gene. *RBTN2* encodes a protein comprising two cysteine-rich LIM domains which are zinc-binding finger like motifs. These play a role in protein-protein interaction since RBTN2 has been found to interact with the basic-khelix-loop-helix protein TAL1. Transgenic mice expressing *RBTN2* develop T cell lymphomas with associated leukaemia with a mean latency of about 8-9 months and exhibit a defined pre-leukaemic phase. The tumour cells can readily be adapted to tissue culture and these can be transplanted in syngeneic mice where they behave as aggressive tumours. To obtain information about the normal role of rbtn2, null mutations have been introduced into the mouse gene to assess the phenotypic effect of lack of this LIM protein. The observed effect is on erythropoiesis which is blocked at an early stage of differentiation. Both Rbtn2 and Tal1 are expressed in erythroid cells and presumably their roles are mediated by the ability to dimerise with each other. This will be discussed.

*HOX11* is a gene activated by the translocation t(10;14)(q24;q11) and the variant (7;10)(q35;q24) which involve T cell receptor gene δ and β respectively. *HOX11* encodes are protein which has a homeodomain which can recognise specific DNA sequences and the HOX11 protein can transactivate transcription. Normally homeodomain genes are essential for developmental regulation and such a normal role for HOX11 seems likely. Null mutations of *hox11* in mice cause asplenia while other sites of expression appear normal. The role of Hox11 in spleen development will be discussed.

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# THIRD SESSION:
# HEMATOPOIETIC CELL SIGNALLING

Chairperson: Moshe Oren

## Normal and leukemic erythropoiesis: Cooperation of receptor tyrosine kinases with members of the steroid/thyroid hormone receptor superfamily

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Self renewal is defined as the ability of progenitor cells to proliferate without entering a differentiation pathway. In the hematopoietic system, self renewal is thought to be restricted to the pluripotent stem cell. In many leukemias, cells with the properties of committed progenitors self renew, but this is thought to be an abnormal property caused by the mutations responsible for leukemic transformation.

Recently, we have developed methods to cultivate normal erythroid progenitors of the chicken *in vitro*. These cells (SCF/TGFα progenitors) coexpress the c-Kit receptor tyrosine kinase and the avian epidermal growth factor receptor (c-ErbB) and undergo continuous self renewal in response to the c-ErbB-ligand (transforming growth factor (TGF) α and estradiol. The common erythroid progenitors, termed SCF progenitors or BFU-E / CFU/E, express c-Kit but only undergo a limited number of cell divisions in response to the c-Kit ligand, stem cell factor (SCF). Both progenitors faithfully reproduce terminal erythroid differentiation in vitro when exposed to differentiation factors.

Firstly, we tried to determine the developmental origin of these self-renewing erythroid progenitors. We could show that SCF progenitors can develop into SCF/TGFα progenitors. This developmental conversion requires 10-14 days and is accompanied by a gradual upregulation of bioactive c-ErbB. Using sera depleted of endogenous growth factor activity, we demonstrated that the development of SCF progenitors into SCF/TGFα progenitors absolutely requires the simultaneous presence of SCF, TGFα and estradiol and is strongly enhanced by an unknown activity in chicken serum. Our results suggest that the self renewal of leukemic cells may reflect a normal potential of certain committed progenitor cells and not, as is presently thought, an abnormal property of leukemic cells that is not shared by the respective normal progenitors.

Secondly, we sought to elucidate the mechanism of action of the v-ErbA oncoprotein encoded by the avian erythroblastosis virus (AEV). AEV transforms erythroblasts through a cooperation between the receptor tyrosine kinase oncogene v-*erb*B and the v-*erb*A oncogene, which represents a mutated nuclear receptor for thyroid hormone (TR-α). In analogy, the chicken epidermal growth factor receptor TGFαR/c-ErbB seems to similarly cooperate with the estrogen receptor (ER) in normal SCF/TGFα progenitors. Since it was shown earlier that v-ErbA interferes with erythroid differentiation, we investigated whether ligand activation of the ER had a similar effect. It was found that estradiol decreases the expression of *gata*-1, *scl*-1, *band*-3, α and β-*globin*, genes, which are normally upregulated during terminal differentiation. Analysis of the effect of various ER mutants showed that this repression does not require the N-terminal transactivation domain (AF-1) nor an intact DNA binding domain. However, mutation of the C-terminal transactivation domain (AF-2) abolishes the effect, which suggests that the ligand activated ER may inhibit differentiation by binding transcriptional coactivators to its C-terminal transactivation domain. As a result, these coactivators may not be available for other transcription factors that regulate erythroid differentiation.

The functional analogy between the ER and v-ErbA further prompted us to investigate whether v-ErbA could sustain self-renewal of primary erythroblasts in the absence of a ligand-activated ER. Indeed, introduction of v-ErbA in chicken bone marrow cells resulted in the outgrowth of erythroid cells in the presence of either SCF or TGFα even when the ER was completely inactive in presence of an ER antagonist. However, there are clear differences between the constitutive actions of v-ErbA and the ligand activated function of the ER. Estradiol-dependent, long-term erythroid self-renewal occurs only in erythroid precursors that express both TGFαR/c-ErbB and cKit, the receptor for SCF. V-*erb*A, on the other hand, promotes sustained self renewal of erythroid precursors that express c-Kit only. In absence of v-ErbA, these progenitors are unable to self renew in SCF plus estradiol.

Interestingly, the v-ErbA expressing normal progenitors differentiated in the presence of differentiation factors (Epo plus insulin), suggesting that v-ErbA is unable to arrest differentiation in these progenitors. This however, turned out not to be true, since a combination of SCF with Epo plus insulin still promoted a slightly delayed differentiation in normal progenitors, but completely arrested differentiation when the normal progenitors expressed v-ErbA. Thus, efficient repression by v-ErbA of erythroid differentiation and gene expression seems to require signaling from the receptor tyrosine kinase c-Kit, suggesting a mechanism through which oncogene cooperation between v-erbB and v-ErbA could occur.

## AP-1 COMPLEX IN LEUKOCYTE DEVELOPMENT AND SIGNALING

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Cell differentiation and programmed cell death constitute pivotal aspects of cellular development and both are active processes requiring gene induction. Thus, we have examined whether transcriptional factors, such as AP-1, are induced in the early phases of these processes in human leukocytes.

We have found that commitment of the human promyelocytic HL-60 cells towards the monocytic/macrophage lineage, upon addition of distinct differentiation agents, is associated with increases in the binding activity of transcription factor AP-1 (1). This increase in AP-1 binding activity was accompanied with increases in c-*fos*, c-*jun*, *jun* B and *jun* D mRNA levels (1,2). In contrast, when HL-60 cells were treated with dimethylsulfoxide or retinoic acid, two granulocytic differentiation inducers, the basal AP-1 binding activity was either drastically reduced or unaffected, respectively (1). Furthermore, the major components of AP-1, c-*fos* and c-*jun*, were not induced during the granulocytic differentiation, and only a weak increase in *jun* B and/or *jun* D mRNA levels were detected (1,2). These data indicate that formation of AP-1 transcription factor is not required for the induction of HL-60 differentiation towards granulocytes, whereas induction of monocytic differentiation is correlated with an increase in AP-1 activity. Thus, the differential expression of AP-1 activity may be critical in the myeloid differentiation towards monocytic or granulocytic lineages.

We have recently found that the ether lipid analogue 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-$OCH_3$) induces rapidly apoptosis in human leukemic HL-60 and U937 myeloid cells and in human leukemic Jurkat T lymphoid cells (3,4). Concomitantly, addition of ET-18-$OCH_3$ to these human leukemic cells induced an increase in the mRNA levels of *fos* and *jun* proto-oncogenes (4). These elevated *fos* and *jun* mRNA levels were correlated with the activation of the AP-1 transcription factor after addition of ET-18-$OCH_3$ to these cells, as assesssed by an enhanced binding activity of transcription factor AP-1 to its cognate DNA sequence as well as by stimulation of transcription from an AP-1 enhancer element (4). Additional agents inducing apoptosis in these leukemic cells promote induction of *fos* and *jun* proto-oncogenes. These data suggest that AP-1 transcription factor can play a role in the promotion of apoptosis in these cells.

Recent evidence shows that phospholipase D (PLD) stimulation constitutes a main signaling pathway in leukocyte activation. High levels of activatable PLD in human myeloid (U937 and HL-60) and T lymphoid (Jurkat) cells have been detected (5). In order to examine the possibility that PLD stimulation may regulate

activation of transcription factor AP-1 in leukocytes, we have trasfected human T lymphocyte Jurkat cells with a plasmid containing an AP-1 enhancer element and a chloramphenicol acetyltransferase reporter gene. We have found that addition of phosphatidic acid (PA), the physiologic product of PLD action on phospholipids, leads to activation of transcription factor AP-1 in Jurkat cells (5). The increases in AP-1 enhancer activity induced by PA were abrogated by the presence of propranolol, an inhibitor of PA phosphohydrolase and protein kinase C (PKC), as well as by the presence of PKC inhibitors or by PKC down-regulation (5). These data indicate that PLD stimulation can activate the transcription factor AP-1 in T lymphocytes, and suggest that the induction of AP-1 enhancer activity by PA is mediated via PKC stimulation. Preliminary experiments indicate that similar results are obtained in other cell types. Thus, these results point out a cross-talk between the generation of PLD-derived lipidic second messengers, which affect processes occurring in the plasma membrane and cytosol leading to early cellular responses, and a nuclear third messenger (AP-1 transcription factor), which affects nuclear events and long-term cellular responses. Furthermore, we have found that induction of AP-1 enhancer activity by stimulation of the TCR/CD3 complex, the key recognition and activation element during physiologic T lymphocyte responses, is mediated through PLD activation (5).

These data indicate that AP-1 transcription factor is activated during cell differentiation, cell death and cell activation in human leukocytes. Experiments are ongoing to examine the role and composition of AP-1 in these processes, as well as to determine whether distinct AP-1 or additional specific genes are engaged in the induction of a particular cellular process in human leukocytes.

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GENE REGULATION BY GROWTH FACTORS

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Growth factors regulate genetic programs which control cell proliferation and differentiation. We have studied this control using several systems, including fibroblasts and the PC12 model neuronal cell line, which responds to nerve growth factor (NGF) by changing from a chromaffin cell- to a sympathetic neuronal cell- phenotype. The changes in PC12 cells include extension of neurites, commencement of electrical excitability and cessation of growth. We will describe our studies of the molecular mechanisms which control these changes.

Signaling by NGF in PC12 starts with NGF binding to *trk*, a receptor tyrosine kinase, which activates the Ras-Raf-map kinase pathway. Three classes of genes are induced: 1) the immediate early, including c-*fos* and c-*myc*; 2) the delayed early, including the tyrosine hydroxylase gene, which is representative of genes whose expression is controlled by neuron activity; and 3) the late genes, including the gene for peripherin, a peripheral-neuron specific intermediate filament protein, and genes encoding other structural components of the neuron. While the pathways which control the first two classes of genes may be readily related to the activation of the *trk* receptor, the mechanisms of the changes which occur with late kinetics are less clear. These late gene regulatory events coincide with the morphological changes in PC12 cells and the gradual cessation of growth.

We have analyzed two cellular systems controlled by growth factors which regulate differentiation and growth arrest. The first is the Myc-Max system. c-Myc is a nuclear phosphoprotein whose expression is correlated with cell proliferation and the block of expression of differentiated functions. Myc forms a heterodimeric partner with Max which binds to E box Myc sites (Ems), at which Myc can act as a transactivator of transcription of genes such as the ornithine decarboxylase gene. We have used fibroblasts to investigate a second activity of Myc, the ability to repress transcription through actions exerted at the core promoters of susceptible genes. We show that the target of repression is the initiator, an element within the core promoter, and that repression depends upon Myc box II, a region of the Myc N terminus which is conserved amongst different Myc family members. We present evidence that a Myc box II-dependent mechanism can repress differentiation-specific genes as well as

genes which arrest cell growth. The latter control provides a basis for Myc's action in stimulating cell proliferation.

We have employed PC12 cells to analyze the effects of NGF on a second growth regulatory system, the cell cycle machinery. We show that NGF represses the activities and levels of multiple *cdk* kinases through induction of a kinase inhibitor and through decrease in kinase protein levels. NGF treatment of PC12 also leads to the disruption of complexes of the transcription factor E2F with the retinoblastoma protein-related factor p107. Surprisingly, NGF strongly induces expression of the $G_1$ phase cyclin, cyclin D1. We discuss the possible role of cyclin D1 in cell cycle check point control and the arrest of cell proliferation.

Together, these studies show that growth factors can have multiple effects on gene activity that can cause profound changes in cell differentiation and proliferation. We will discuss how coordinate changes in these systems may regulate neuronal differentiation.

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## REGULATION OF THE IFN SYSTEM AND CELL GROWTH BY IRF-1 AND IRF-2

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Regulation of cell growth and differentiation is mediated by a variety of extracellular agents such as cytokines. One class of cytokines is the interferons (IFNs) which exhibit many biological activities including anti-proliferative activity. In fact, IFNs exhibit anti-proliferative activity on many normal and transformed cells, and can block growth factor-stimulated cell cycle transitions. In the process of analyzing the mechanism of the IFN-β gene expression, we have identified two specific nuclear factors, IRF-1 and IRF-2, which interact specifically with the IFN-β promoter. These factors are structurally related, particularly in their amino-terminal regions which provide DNA binding specificity, and they bind independently to promoter elements within the *IFN-α* and *IFN-β* genes as well as many IFN-inducible genes, by recognizing the consensus sequence motif, G(A)AAA$^{G}/_{C}{}^{T}/_{C}$GAAA$^{G}/_{C}{}^{T}/_{C}$. Evidence has been provided showing that viral infection results in an increase in IRF-1 synthesis and the activation of some kinase-dependent signaling event, both of which are required for efficient activation of the IFN-β promoter.

In the context of the regulation of cell growth by IFNs, it is interesting that the IRF-1 gene is IFN-inducible per se. Hence it may be one of the target genes for the anti-proliferative function of IFNs. Recently, we have provided evidence that restrained cell growth depend upon the balance between these two competitive factors. The IRF-1/IRF-2 expression ratio results in profoundly altered growth properties of NIH3T3 cells. In fact, when the IRF-2 gene was overexpressed in NIH3T3 cells, the cells became transformed and had enhanced tumorigenicity in nude mice. This transformed phenotype was reversed by concomitant overexpression of the IRF-1 gene . Thus, restrained cell growth depends on a balance between these two mutually-antagonistic transcription factors. These findings suggest a novel mechanism in the regulation of cell growth and imply the involvement of the two mutually antagonistic transcription factors in oncogenesis.

In order to gain further insights on the role of the IRFs, mice deficient in either IRF-1 or IRF-2 or both have been generated. It was shown that embryonic fibroblasts (EFs) from mice with a null mutation in the IRF-1 gene (IRF-1$^{-/-}$ mice) can be transformed by expression of an activated c-Ha-ras oncogene. The transformed phenotype of ras-expressing IRF-1$^{-/-}$ EFs could be suppressed by the expression of the IRF-1 cDNA. Furthermore, expression of the c-Ha-ras

oncogene causes wild-type but not IRF-1$^{-/-}$EFs to undergo apoptosis when combined with a block to cell proliferation or treated by anticancer drugs of ionizing radiation. We also provided evidence for the possible involvement of the IRF-1 gene deletion and/or inactivation in human MDS/leukemia. Currently, we are searching for the target gene(s) of IRF-1 which functions in the negative regulatin of cell growth.

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# FOURTH SESSION:
# MOLECULAR MECHANISMS OF DIFFERENTIATION

Chairperson: Alan Bernstein

**DIFFERENTIATION PRIMARY RESPONSE GENES AND PROTO-ONCOGENES AS POSITIVE AND NEGATIVE REGULATORS OF TERMINAL HEMATOPOIETIC CELL DIFFERENTIATION**

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We have surmised that the molecular engine which drives the differentiation process forward involves induction of positive regulators of terminal cell differentiation, which may be found among hematopoietic differentiation primary response (MyD) genes, as well as suppression of negative regulators, which may be found among genes which control hematopoietic cell growth. The talk will focus on how this line of thought has lead us to identify some of the players which regulate terminal hematopoietic differentiation, growth arrest and apoptosis, encompassing both positive and negative regulators, and how, once identified, we were able to manipulate the expression of these genes in either leukemic or normal hematopoietic precursor cells to study the mechanisms that control terminal differentiation, growth arrest and programmed cell death and loss\acquisition of leukemogenicity.

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The involvement of the transcription factor PU.1 in the development of both lymphoid and myeloid cells. Richard Maki, Scott McKercher, Karen Anderson, Greg Henkel, Deborah Vestal, and Bruce Torbett[1], La Jolla Cancer Research Foundation, La Jolla, CA 92037, and Scripps Clinic and Research Institute, La Jolla, CA. 92037[1]

The transcription factor PU.1 is a DNA binding protein that is expressed in B cells and myeloid cells. The PU.1 protein binds to a purine rich sequence containing the core sequence 5'-GGAA-3'. Evidence has been obtained suggesting that PU.1 regulates the expression of several genes including c-fms, CD11b, J chain and both light and heavy chain immunoglobulins. The PU.1 protein belongs to a family of transcription factors that are related by a high degree of sequence similarity in the DNA binding domain of these proteins. This family has been called the ETS family of transcription factors.

Recently, we have disrupted the gene for PU.1 in the mouse to determine its role in the development of cells in the immune system. Mice homozygous for the disruption of the PU.1 gene are born but die within 24 to 48 hours after birth due to septicemia. We are investigating ways in which we can protect the animals and extend their lives. An analysis of the blood from these animals has revealed that the animals are devoid of macrophages, granulocytes, neutrophils and lymphocytes. We do see erythrocytes and megakaryocytes, however. When we looked at tissues including liver, bone marrow, spleen or thymus, we did not see any macrophages by either immunostaining with F4/80 or staining for esterase activity. We have looked for mature B cells in the spleen but have not found any evidence that a population of surface Ig positive cells are present. We have looked for Ig rearrangement but have not found evidence for this. We have seen a population of B220 positive cells in the spleen by FACS analysis however, that may suggest that B cells are able to differentiate part way down the B cell pathway but then are blocked. Interestingly, we do not see any mature T cells in the thymus of these animals. This is interesting since we have not detected expression of PU.1 in any T cell line that we have looked at. It is possible that an early precursor to T cells expresses PU.1 and then the expression is turned off. We are currently examining how these results fit into present models for development in the hematopoietic system.

## REGULATION OF MAX/MYC/MXI1 NETWORK DURING DIFFERENTIATION AND APOPTOSIS OF HUMAN MYELOID CELLS

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While the expression of c-myc is extensively regulated, no major changes in max expression have been reported so far with respect to differentiation. We have studied the expression of c-Myc, Max and Max-interacting proteins Mad and Mxi1 during *in vitro* differentiation of the bipotent human myeloid leukemia K562 cell line. This cell model system allowed us to compare c-Myc and Max expression during differentiation along erythroid (induced by 1-β-D-arabinofuranosylcytosine, ara-C) and myelomonocytic lineages (induced by TPA or staurosporin). We have previously found that c-myc down-regulation is not obligatory for proliferation arrest of K562 cells but was down-regulated with terminal differentiation induced by ara-C or TPA. We have analyzed the expression of the c-myc network genes along both differentiation pathways.

The expression level of max remained unchanged during TPA- and staurosporine-induced myelomonocytic differentiation. In contrast, max mRNA and protein were down-regulated during ara-C-induced erythroid differentiation of K562 cells, thus demonstrating that max gene is regulated during cell differentiation. mxi1 expression slightly increased during ara-C-induced erythroid differentiation but was strongly down-regulated during TPA-induced myelomonocytic differentiation of K562. Expression of mad did not change with erythroid differentiation and increased during induction of the myelomonocytic pathway.

We have obtained K562 sublines stably transfected with a zinc-inducible c-myc gene. In these clones the overexpression of c-Myc did not interfere with TPA-induced myelomonocytic differentiation. In contrast, erythroid differentiation was significantly inhibited upon c-myc induction despite concomitant down-regulation of max expression. These results suggest a differential role for c-Myc in the human myeloid cell differentiation depending on the cell lineage and that c-Myc has some Max-independent activity in K562 cells.

Treatment of the human myeloid leukemia K562 cells with the serine/threonin protein phosphatase inhibitors okadaic acid (OA) and calyculin A resulted in down-regulation of both c-myc and max genes, observed at the steady-state levels of both mRNA and protein. The extent of the down-regulation was dependent on the dose and on the treatment time. Interestingly, the expression of both genes returned to control levels after the removal of the okadaic acid from the media. OA also induced mitotic arrest followed by apoptosis in K562 cells. Apoptosis was confirmed by other criteria as oligonucleosomal DNA fragmentation and *in situ* end-labeling. We found that c-myc and max down-regulation depended on the presence of OA in the medium but

it is neither a consequence of the mitotic arrest nor of the apoptosis. In K562 sublines transfected with a conditionally expressing c-myc gene, c-myc overexpression had no effect on the apoptotic response to okadaic acid. Interestingly, all these effects were observed at OA concentrations that inhibited the activity of protein phosphatase type 2A but not type 1. PP2A activity recovers upon withdrawal of OA from the media. We conclude that in K562 cells the inhibition of protein phosphatase type 2A is associated to down-regulation of c-myc and max genes. The okadaic acid-mediated mechanism of c-myc and max down-regulation and the participation of c-myc and other apoptosis regulatory genes during K562 apoptosis are currently under investigation.

This work is supported by grant PB92-506C02-2 from DGICYT and by a grant from Fundación Ramón Areces.

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**Expression and putative roles of the transcription factor c-Ets1.**

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In the early embryo, the development of the hemopoietic stem cell and endothelial cell lineages are tightly related. The morphological features of the early stages of embryogenesis suggest the existence of hemangioblasts as common precursor to these two lineages. In the blood islands of the extraembryonic area, both endothelial and hemopoietic cells differentiate from seemingly homogeneous cells aggregates ; peripheral cells give rise to the endothelial envelope while central cells become the primitive erythroid cells (reviewed in Dieterlen-Lièvre, 1994). Several genes that are expressed in these blood islands have been also detected in endothelial cells during angiogenesis or vasculogenesis : they include the SCL/TAL1 gene encoding an helix-loop-helix transcription factor (Kallianpur et al., 1994), the flk1 gene encoding a VEGF high affinity receptor (Millauer et al., 1993) and the tek gene encoding another endotheliai-specific tyrosine kinase (Dumont et al., 194). Conversely transcripts for c-myb that is expressed in hemopoietic cells are also detected in these blood islands (Quéva et al., 1992).

Using the *in situ* hybridization technique, we have demonstrated that the c-ets1 gene is expressed in these blood islands as well as in endothelial cells during angiogenesis in mouse and chicken embryos. This expression is no longer detected in blood vessels irrigating healthy adult tissues. However, when angiogenesis resumes in developing human tumors, the expression of c-ets1 is induced in endothelial cells of capillaries irrigating the tumor (Vandenbunder et al., 1989; Wernert et al., 1992).

The c-ets1 proto-oncogene is the cellular progenitor of the viral v-ets that is associated with v-myb in the genome of the avian leukemia virus E26 (Leprince et al., 1983; Nunn et al., 1983). c-Ets1 is the founder of a new family of transcription factors that share a conserved sequence of about 85 amino acids, the DNA binding domain, and recognize target sequences containing a common GGAA/T motif (reviewed in Laudet et al., 1993). The most closely related members of this family, c-ets1, ets2, erg and fli, have also homologies in their transactivating domain, and it is likely that they take part in the regulation of overlapping sets of genes.

During early embryogenesis the expression patterns of c-ets1, erg and fli superimpose in various mesodermal derivatives, such as the blood islands, and in neural crest cells. The differences between the regulation of c-ets1, ets2, erg and fli genes expression and the putative roles of these transcription factors in hemopoietic versus non hemopoietic cells will be discussed.

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**The Myc Protein Network and Cell Differentiation**

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The c-Myc protein associates with Max and is a transcriptional activator. Myc has been implicated as a key regulator of the differentiation programs of a variety of cell types. The Mad protein is a bHLHZip protein which appears to antagonize the function of Myc in three ways: (i) Mad competes with Myc for binding to Max; (ii) Mad:Max competes with Myc:Max for binding to DNA target sites, and (iii) Mad:Max acts as a transcriptional repressor wheras Myc:Max is an activator. We have observed a switch from Myc:Max to Mad:Max complexes upon differentiation of myeloid cells. Recently we have shown in epidermal keratinocytes *in situ* and *in vitro* that Myc levels are rapidly downregulated as they differentiate. In contrast, Mad protein is induced as these cells differentiate and appears to be expressed in the growth-arrested suprabasal spinous and granular cell layers. Using HPV18 transformed human keratinocytes and a HPV16 transgenic mouse model system, we find Mad expression is lost in cells that can no longer differentiate in culture and is found only at sites of residual differentiation in tumors of transgenic mice. These results suggest that Mad may play a fundamental role in the terminal differentiation of epidermal keratinocytes and that the stratification of Myc and Mad expression is maintained even in malignant tumors. Furthermore, these results raise the possibility that the disruption of the *mad* locus or loss of proper *mad* regulation may be involved in tumorigenesis of these cells.

In an attempt to identify other Max-binding proteins that may impinge of the regulation of Myc, we used a two-hybrid approach employing Max as "bait". In this screen we identified four novel bHLHZip proteins that interact with Max. Two of these proteins are related to Mad and Mxi and the other two are quite distinct from either Myc or Mad. The potential relationship of these proteins to proliferation and differentiation was explored using *in situ* hybridization of mouse embryos and adult tissues. Results of these experiments and initial molecular characterization of these new bHLHZip proteins will be presented. Taken together our findings suggest that the biological effects of Myc are modulated through an expanding network of activating and repressing transcription factors.

# FIFTH SESSION:
# CONTROL OF PROLIFERATION AND APOPTOSIS

Chairperson: Jerry Adams

## THE INTEGRATED CONTROL OF CELL PROLIFERATION AND PROGRAMMED CELL DEATH (APOPTOSIS) BY ONCOGENES

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The spontaneous outgrowth of more rapidly proliferating mutant cells is a source of continuous neoplastic risk to multicellular organisms. Nonetheless, metazoans require proliferation of their component cells in order to differentiate, maintain and repair their morphologies. There thus exists a dynamic natural selective tension between an organism and its component cells. Metazoans must somehow permit proliferation of their component cells yet a the same time rigorously suppress outgrowth of neoplastic variants.

The c-*myc* proto-oncogene encodes an essential component of the cell's proliferative machinery and its deregulated expression is implicated in most neoplasms. Intriguingly, c-*myc* is also a potent inducer of apoptosis in cells deprived of growth factors or subjected to cytostatic drugs. Myc-induced apoptosis is dependent upon the level at which c-Myc is expressed, and deletion mapping shows that regions of c-Myc required for apoptosis overlap with regions necessary for co-transformation, autoregulation, inhibition of differentiation, transcriptional activation and sequence-specific DNA binding. Moreover, induction of apoptosis by c-Myc requires association with c-Myc's heterologous partner, Max. All of this strongly implies that c-Myc drives apoptosis through the transcriptional modulation of target genes.

Two simple models can be invoked to explain the induction of apoptosis by c-Myc. In one, death arises from conflict generated by the inappropriate or unscheduled expression of c-Myc under conditions that would normally promote growth arrest. In this "Conflict" model, induction of apoptosis is a not a normal function of c-Myc but a pathological manifestation of its deregulation. The other model holds that induction of apoptosis is a normal obligate function of c-Myc that is usually suppressed by the action of specific survival factors. We term this the "Dual Signal" model - one signal activates both mitogenesis and apoptosis, the second is required to block cell death.

Our studies show that c-Myc-induced apoptosis in fibroblasts is specifically suppressed by the insulin-like growth factors and PDGF independently of, growth state, position within the cell cycle, or whether apoptosis is triggered by low growth factors or by DNA damage. These results favour the Dual Signal model.

c-Myc-induced apoptosis is also suppressed by expression of the *bcl-2* proto-oncogene and we have recently identified novel members of the *bcl-2* family, some of which trigger apoptosis rather than suppress it. c-Myc-induced apoptosis also requires expression of p53.

The various ways in which these various pro-apoptotic and anti-apoptotic factors interact and influence each other will be discussed.

**Spi-1/PU.1 overexpression induces erythroid tumors in transgenic mice.**

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The Spleen Focus Forming Virus (SFFV) induces in mice an acute erythroleukemia which progress as a multistep disease (for review:(1)). The early step is characterized by the massive infection of target cells for SFFV transformation *i.e.* an immature erythroid cell highly sensitive to the erythropoietin (EPO), most probably a late erythroid burst-forming unit (BFU-E) or an erythroid colony-forming unit (CFU-E) progenitor. This results in the polyclonal expansion of immature proerythroblasts that differentiate into abnormal erythrocytes. The pathogenic properties of SFFV are due to the glycoprotein encoded by its env gene (gp55). In case of SFFVp (the polycythemic strain of SFFV), the gp55 is partially processed in a highly glycosylated form which is anchored at the cell membrane. The interaction between the transmembrane domain of the gp55 and the extracytoplasmic and transmembrane domains of the EPO receptor constitutively activates the EPO receptor and would be responsible of the abrogation of the EPO requirement of the SFFVp-infected erythroid cells. The later step is characterized by the emergence of tumorigenic proerythroblasts blocked in their differentiation program. These Friend tumor cells are clonal with respect to alterations of two cellular genes *spi-1* and p53. Consequently, these 2 genes have been presumed to be involved in the malignant transformation of the proerythroblasts. The genetic alteration of p53 gene occurs by various processes as point mutations, allelic deletions and insertional mutagenesis, all of them leading to the loss of expression of the normal p53 protein. The *spi-1* gene is mutated by integration of the SFFV provirus in the vicinity of the gene (2). Most probably, the transcriptional enhancer elements supplied by SFFV LTR are involved in the large overexpression of the normal *spi-1* gene which is observed in all Friend tumor cells.

The *spi-1* gene encodes the hematopoietic transcription factor PU.1, a member of the Ets family (3). It recognizes a purine rich sequence motif around a minimal central core consensus 5'-G/AGAA-3' in the transcription regulatory regions of genes. It is normally expressed in all hematopoietic cell lineages except in T cell lineage. As could be anticipated from this expression pattern, an increasing number of functional motifs Spi-1/PU.1 has been identified in various transcriptional promoters/enhancers of genes expressed in myelomonocytic cells, mast cells and B lymphoid cells (for review:4). The phenotype of PU.1 negative homozygotes embryos is consistent with these multilineage effects. They present a multilineage defect characterized by a defective development of progenitors in monocytes, granulocytes as

well as B and T lymphoid lineages (5). Thus, all these data, suggest that Spi-1/PU.1 may participate to the hematopoietic development as a basal transcriptional regulator. However Spi-1/PU.1 would not be sufficient to regulate the expression of lineage-specific differentiation markers.

During the Friend disease, the multistep progression of the SFFV-infected proerythroblast towards malignancy appears to result from interactive processes between viral (gp55) and cellular proteins (at least p53 and Spi-1/PU.1) . Thus to discern the effective role of *spi-1* in the transformation of proerythroblast, we generated *spi-1*-transgenic mice. As our goal was to approach the role of *spi-1* in erythroleukemogenesis, the transcription of the transgene was driven by the SFFV LTR which contains transcriptional enhancer elements active in hematopoietic cells *in vitro* and *in vivo* , and is naturally involved in *spi-1* deregulation. In this report we show that SFFV LTR-*spi-1*-transgenic mice present disorders specifically in the erythroid lineage. Diseased transgenic mice are anemic and develop a large hepatosplenomegaly which is fatal within 3.6±1.5 months. Histological, cytological and molecular analyses reveal that spleen and liver enlargement is due to the proliferation of a population of immature erythroid cells which are dependent on erythropoietin for their growth. They are blocked in their differentiation and are not tumorigenic in Nude recipient mice. Additional genetic alterations seem necessary for the emergence of tumorigenic *spi-1*-transgenic proerythroblasts which are independent on erythropoietin for their proliferation *in vivo* and *in vitro*.

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## THE INVOLVEMENT OF p53 IN THE CONTROL OF CELL FATE: TRANSCRIPTIONAL AND NON-TRANSCRIPTIONAL MECHANISMS.

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The p53 gene is a major target for genetic alterations in human cancer. Such alterations usually eliminate the normal tumor suppressor function of the wild type (wt) protein, which presumably serves as a barrier to tumor progression.

Reconstitution of wt p53 activity in various types of transformed cells revealed that overexpression of the normal protein can affect cell fate in a number of ways. While in some cases wt p53 activity causes a viable growth arrest, typically in G1, it can induce apoptotic cell death in other instances. The first demonstration of the involvement of p53 in apoptosis came from the study of M1 mouse myeloid leukemia cells, stably transfected with a temperature sensitive (ts) p53 mutant[1]. Induction of wt p53 activity at 32°C in such cells (LTR6 clone) caused a vigorous and efficient apoptotic response. Interestingly, this response was markedly inhibited by IL-6.

Examination of cell cycle parameters revealed that despite the presence of high levels of p53 activity, reflected also by induction of the Waf1 gene, the cells did not exhibit any evidence for a G1 arrest[2]. Rather, they proceeded directly from an active cell cycle into apoptosis. On the other hand, LTR6 cells rescued from apoptosis by IL-6 were very efficiently arrested in G0/G1[3]. This observation raised the possibility that, at least in these hematopoietic cells, a failure to arrest properly in G1 in response to the activation of p53 may promote apoptotic cell death; conversely, one could propose that under conditions where p53 can establish an effective G1 arrest, cells will become less prone to p53-mediated apoptosis.

The discovery of the activation of the Waf1 gene by p53, and the subsequent demonstration that this process is important for the induction of p53-mediated G1 arrest, have strongly suggested that this type of growth arrest is obtained through the functional activation of the pRB protein, as a result of its reduced phosphorylation in cell expressing high levels of $p21^{waf1}$. Furthermore, evidence based on the study of DNA tumor viruses, as well as of certain human tumors, raised the possibility that failure pRB family proteins to function properly might trigger p53-mediated apoptosis. It was therefore of interest to find out whether the status of pRB in a given cell could determine the fate of the cell when exposed to high levels of wt p53 activity. To that end, we took advantage of a transient transfection assay in HeLa cells, where wt p53 has been shown to induce apoptosis[4,5]. Expression vectors for pRB, either wt or functionally-impaired, were co-transfected with wt p53 expression plasmids, and the rate of apoptosis was monitored. The results showed clearly that excess functional pRB could reduce significantly p53-mediated apoptosis, presumably by converting it into a viable growth arrest. Hence, pRB can serve as a modulator of cell fate in the presence of activated wt p53.

While pRB could block efficiently p53-mediated apoptosis in HeLa cells when p53 was expressed under the control of the MLV LTR, its effect was rather marginal when wt p53 expression was driven by the stronger CMV promoter. One possible interpretation could be that higher levels of wt p53 trigger an alternative apoptotic pathway, which is refractory to the effect of RB. It is conceivable that such putative pathway may promote apoptosis even when cells are G1 arrested.
To further explore the idea of alternative p53-dependent apoptotic pathways, we tested the ability of various mutant forms of p53 to induce apoptosis in HeLa cells. Surprisingly, we found that a highly deleted form of p53, lacking much of its sequence-specific DNA binding domain, could still act as a potent apoptotic inducer. Transactivation assays confirmed that this mutant has totally lost the ability to activate a p53-responsive promoter. Hence, there indeed appears to exist an alternative pathway for p53-mediated apoptosis, which does not rely on the ability of p53 to activate specific target genes. The implications of this observation will be discussed.
The ability of IL-6 to rescue M1 cells from p53-mediated apoptosis also raised the possibility that the activity of p53 renders hematopoietic cells more dependent on survival factors. To test this possibility, we took advantage of the DA-1 mouse lymphoma-derived cell line. DA-1 cells appear to possess functional wt p53, and are strictly IL-3 dependent. Retroviral vectors were employed in order to introduce into DA-1 cells a negative dominant p53 mutants. Analysis of the transcriptional activity of the endogenous p53 in the infected cells revealed that it was greatly compromised in the presence of the dominant-negative mutants. As expected, such cells were remarkably refractory to the induction of apoptosis by ionizing radiation. Importantly, they were protected to an almost comparable extent from apoptosis induced by IL-3 deprivation[6]. Hence, wt p53 does play a role in mediating apoptotic death in at least some types of hematopoietic cells deprived of survival factors. Most notably, while cells deficient in p53 function exhibited extended viability in the absence of IL-3, they did not continue to proliferate under these conditions. Thus, whereas the survival aspect of IL-3 appears to be p53-related, the proliferative effects of this cytokine are probably unrelated to p53.

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**Screening for target genes of the c-Myc/Max transcription factor**

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The c-Myc oncoprotein (Myc) and its partner Max function as a heterodimeric, sequence-specific transcriptional activator. Several lines of evidence indicate that this activity is required for Myc to induce cell proliferation, apoptosis and/or malignant transformation. It is thus widely accepted that these cellular phenotypes involve direct regulation by Myc/Max of key target genes, most of which remain to be identified (for review, ref. 1). As an experimental system to selectively control Myc activity in cells, we use a hormone-dependent Myc-oestrogen receptor hybrid protein (MycMER). Such a chimaera has been used before in immortalized rodent fibroblast lines (e.g. Rat1, NIH-3T3) to isolate and/or characterize those target genes identified to date (1). However, with few exceptions, Myc does not fully transform those cell lines and the ill-characterized immortalization process itself may well have led to prior deregulation of Myc target genes. Seeking a system that would mimic more closely the effects of Myc in oncogenic transformation of normal cells, we co-transfected primary rat embryo fibroblasts with plasmids encoding MycMER and the v-Ha-Ras oncoprotein. In this system, the appearance of transformed-cell foci is strictly hormone-dependent. Furthermore, most of the clones derived from MycMER+Ras foci show a conditional transformed phenotype (ability to grow in soft-agar, morphology, acidification of medium) and undergo apoptosis in low serum only if MycMER activity is induced. We are currently characterizing these clones by Northern blotting for the regulation of known Myc target genes (α-prothymosin and ODC). In paralel, we are using the technique of differential PCR display to clone cDNAs corresponding to Myc-induced mRNAs. Results from these experiments will be presented.

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## Mediation of c-Myc-Induced Apoptosis by p53

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The cellular proto-oncogene c-*myc* is involved in cell proliferation and transformation, but is also implicated in the induction of programmed cell death (apoptosis). The same characteristics have been described for the tumor suppressor gene *p53*, the most commonly mutated gene in human cancer. We have previously shown that simian virus 40 large T antigen antagonizes c-Myc-induced apoptosis by binding and inactivating wild-type p53 protein in NIH 3T3-L1 mouse fibroblasts (1). To determine the role of p53 in a more direct way we employed a conditional c-Myc protein fused to the estrogen receptor hormon binding domain. In quiescent mouse fibroblasts expressing wild-type p53, activation of c-Myc was found to induce apoptosis and cell cycle re-entry, preceded by stabilization of p53. In contrast, in quiescent p53 null fibroblasts, activation of c-Myc did induce cell cycle re-entry but not apoptosis (2). These results suggest that p53 mediates apoptosis as a safeguard mechanism to prevent cell proliferation induced by oncogene activation.

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# POSTERS

## ABSTRACT

**Prothymosina alpha (PTA) stimulates the phosphorylation of a 105 kD protein, in cellular extracts of cycling but not in quiescent cells.**

**Alvarez, C., Zavilde J.B., Vidal, A., Loidi, L., Carneiro, C., Buceta, M.F., Dieguez, C., Dominguez,F. and Vega, F.V.**

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Phosphorylation and dephosphorylation mechanisms take part during proliferation as well as in the commitments of quiescent cells (Go) to reenter the cycle. PTA has been claimed to be phosphorylated "in vitro" and "in vivo"but is action on the phosphorylation of other proteins has not been reported. Since high levels of PTA are associated to the proliferative state of the cells, the phosphorylation of some others proteins could in turn be dependent on the levels of the former. We have hypothesized that a relationship could exist between PTA levels and the aforementioned regulatory phosphorylation processes. We studied the PTA dependent phosphorylation of proteins in whole cellular extracts, in the searching for a putative phosphorylation/dephosphorylation mechanism that could take part in the regulation of cell cycling in proliferating cells, or the passage of Go to the proliferative state in quiescent cells.

In this work we demostrate the stimulation of the phosphorylation of a 105 kD protein band, in cell extracts derived from different cell types. Cells derived from a human promyelocytic leukemic tumor cell line (HL 60) contain a phosphorylation system stimulated by PTA.

The addition of PTA to cellular extracts obtained from NIH 3T3 quiescent cells did not stimulate the phosphorylation of the 105 kD band. In cells under exponential growth barely phosphorylated band obtained in the absence of PTA, was markedly stimulated in its presence.

Very low concentration of $Ca^{2+}$ or another divalent cation must be necessary for this phosphorylation, since EGTA $10^{-3}$ M abolish and $Ca^{2+}$ at low ($10^{-7}$M) or high ($10^{-4}$M) concentration enhances the PTA effect in proliferating cells.Maximal stimulation is obtained with the combination of PTA plus $Ca^{2+}$.

In NIH 3T3 cells we investigate the stimulation of the phosphorylation of the 105 kD protein in cells coming from quiescence. In cellular extracts obtained from Go (quiescence) the addition of PTA did not stimulates the phosphorylation of the 105 kD band. After 8 hours of addition of 15% serum to these cells the stimulation was obtained.

Cells were also syncronized by double blocking with thymidine and followed along the cell cycle by preparing cellular extracts at different times after releasing from thymidine arrest. In G1-S, S, S-G2, G2-M-G1 and M, the phosphorylation of the 105 kD band was stimulated by PTA. In cycling cells and the presence of PTA the 105 protein is heavily phosphorylated, but there is also a phosphorylation in the absence of added PTA which increases together with the progression of the cells from G1 to M along the cycle.These results indicate that phosphorylation of the 105 kD protein is also produced in the absence of added PTA, probably when endogenous PTA or some other protein is abundant, like during mitosis. These results indicate that some factor, which stimulates the phosphorylation of the 105 kD protein is produced by the cells after leaving quiescence or arrest. At the present time we do not known whether is PTA itself or another protein responsible for this effect.

The stimulus of phosphorylation of the 105 kD protein by PTA open a new insight in the studies of the phosphorylation cascades, which are known to play a central rol in the passage from quiescence to proliferation in these cells.

## LINEAGE-RESTRICTED REGULATION OF THE MURINE SCL/TAL-1 PROMOTER

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Rearrangements of the SCL/TAL-1 gene are the commonest molecular abnormality in T-cell acute lymphoblastic leukaemia (1). The SCL/TAL-1 gene encodes a basic helix-loop-helix transcription factor that is expressed in multipotent haemopoietic progenitors prior to lineage commitment and plays a pivotal role in haemopoiesis (2,3). Its expression is maintained during differentiation along erythroid, mast and megakaryocytic lineages, but is repressed following commitment to non-expressing lineages (4). In order to begin to address the molecular mechanisms underlying this complex pattern of expression, we have cloned the murine genomic SCL locus and analyzed the differential splicing patterns giving rise to two alternative polypeptides (5). Here we present the regulation of the murine SCL promoter in erythroid and T cell lines.

Analysis of the methylation and chromatin structure of the SCL promoter region showed that SCL mRNA expression correlated with the presence of DNase hypersensitive sites and the methylation status. Transient reporter assays demonstrated that promoter 1a was active in erythroid cells but not in T cells. Sequences between -187 and +26 were sufficient for lineage-restricted activity of promoter 1a. A joint promoter construct containing both promoter 1a and promoter 1b also exhibited lineage-restricted activity. Conserved GATA (-37), MAZ (+242) and ETS (+264) motifs were all shown to contribute to SCL promoter activity in erythroid cells but several other motifs were not required for full promoter activity. The pattern of complexes binding to the +242 MAZ and +264 ETS sites were the same in erythroid and T cells. However GATA-1 bound the -37 GATA site in erythroid cells, whereas in T cells GATA-3 was only able to bind weakly, if at all. Moreover GATA-1 but not GATA-2 or GATA-3 was able to transactivate SCL promoter 1a in a T cell environment. These results suggest that inactivity of SCL promoter 1a in T cells reflected absence of GATA-1 rather than the presence of trans-dominant negative regulators.

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# EFFECT OF DIFFERENT CYTOKINES ON "IN VITRO" MEGAKARYOCYTIC DIFFERENTIATION.

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Differentiation and maturation of megakaryocytes leads to platelet formation. This process is characterized by three major events, some of them unique to this cell type: i) endomitosis, ii) expression of platelets' antigens, and iii) cytoplasmic maturation. It has been shown that both lineage and non-lineage specific cytokines could play an active role in megakaryocytic differentiation, although little is known about the transduction signals involved in their activity.
In our lab we are interested in studying the molecular events that take place during these processes. We are using established cell lines shown to undergo megakaryocytic differentiation upon certain stimuli such as phorbol esters treatment or haemopoietic cytokines stimulation. In a first approach we have investigated the effect of TPA, IL3, GM-CSF, IL6, LIF and SCF on the appearance of differentiation feature in HEL and MEG01 cell lines, in particular polyploidization, platelet antigens expression and phenotypic maturation. Results will be presented that show that although TPA is able to induce differentiation of both cell lines, the addition of individual cytokines only affect certain aspects of MEG01s differentiation. We are also focusing in the understanding of the molecular mechanisms responsible for these cells to undergo endomitosis.

**REGULATION OF MXI1 AND MAD DURING DIFFERENTIATION AND APOPTOSIS OF HUMAN LEUKEMIA K562 CELLS**

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The mxi1 and mad genes encode proteins which form heterodimers with Max. Conversely to Myc/Max heterodimers, Mxi/Max and Mad/Max are devoid of transactivation activity. This fact suggests that they can modulate Myc activity by sequestering Max. In order to study the expression of mxi1 and mad genes during differentiation and apoptosis we have chosen the K562 cell line as a model system. Depending on the used chemical agent these cells can be induced to differentiate through erythroid or myelomonocytic pathways. In the other hand, treatment with okadaic acid, a potent protein phosphatase inhibitor, induces apoptosis in this leukemic cell line.

We induced erythroid differentiation of K562 cells using 1-ß-D-arabinofuranosylcytosine (ara-C, 1 µM). After this treatment a slight increase in mxi1 expression was observed while mad expression was unaffected. Myelomonocytic differentiation was induced in these cells by adding 12-O-tetradecanoylphorbol-13-acetate (TPA, 10 nM). The analysis of mxi1 expression in this case revealed a strong down-regulation detected after 6 hours of treatment while mad expression slightly increased. These results demonstrate a mode of mxi1 and mad regulation in K562 which is different from that described for other leukemic myeloid cell lines as HL60 and U937. When apoptosis was induced by okadaic acid (15 nM) the expression of mxi1 and mad was down-regulated.

We are currently obtaining transfectants with mxi1 to study the effects of its ectopic expression in the K562 model system.

# The transcription factor PU.1 is necessary for macrophage proliferation

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PU.1 is an *ets* related transcription factor that is specific for macrophages and B lymphocytes. In serum free media, bone marrow derived macrophages are able to produce colonies. In the presence of M-CSF, proliferation of PU.1 transfected macrophages was enhanced in relation to the controls. A similar effect was observed in the presence of GM-CSF, but not in the presence of IL1, IL2, IL3, IL4, IL6 or G-CSF.

Macrophages transfected with antisense PU.1 showed a decreased response in proliferation to M-CSF in relation to macrophages transfected with the control vector. A decreased proliferation was also observed when antisense oligonucleotides were added to the culture media containing M-CSF.

Mutations by alanines of the serines 41 and 45, that are sites of casein-kinase depending phosphorylation, abrogate the proliferative effect of PU.1.

These experiments demonstrated that the transcription factor PU.1 is necessary for macrophage proliferation and also that phosphorylation of PU.1 is required for the proliferative effect.

## CHARACTERIZATION OF THE PROMOTER REGION OF PECAM-1 GENE

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Platelet endothelial cell adhesion molecule-1 (PECAM-1), is a widely distributed cell adhesion molecule expressed by circulating platelets, leucocytes and vascular endothelial cells. PECAM-1 is a member of the Ig superfamily as other surface antigens such as NCAM-I, ICAM-I ICAM-2 or CEA and mediates the cellular interactions during the process of transendothelial cell migration. We are studying the regulated expression and function of PECAM-1 in monocytic cells (1). In this context, we have been able to isolate a PECAM-1 genomic clone from a λEMBL library using as a probe an oligonucleotide corresponding to the unstranslated 5´ region (GTTGAGAACCCCGCCCAGTGAAAAGC). The HindIII 5kb fragment of this clone containing the putative promotor region (5´ flanking region), was subcloned into the plasmid PBS. From this fragment, several constructs were made into the PXP2 vector containing the luciferase reporter gene. One of these constructs, the 1.6kb PstI/HindIII fragment was found to display promoter activity by transfection in U-937 cells. Then, further constructs of NheI/HindIII 0.9kb, NheI/PstI 0.7kb, BglII/PstI 0.46 kb and 0.2kb NheI/BglII were made and assayed for activity. The maximum activity was found within the 0.2kb fragment. No consensus TATA box has been found, but several putative consensus regions for transcription factors have been identified: 2 GATA, 2 RA, 4 AP2, 1 SP1, 1AP1, 2 NFKB, 1 Imperfect octomer, 1 heptamer, 2 Ets sites, 2 E -Box, 1 PU Box and a glucocorticoid response element (GRE). Interestingly, we find that the PKC activator PMA is able to increase the PECAM-1 promoter activity in some of the constructs. This could be explained by the presence of NFKβ, AP2 and AP1 sites; and corroborates previous studies demonstrating an increased surface expression of PECAM-1 during the PMA-induced macrophage differentiation. (2). Furthermore, we find that 1, 2-dioctanoyl glycerol and 1-octanoyl, 2-acetylglycerol also induce the surface expression of PECAM-1. We are trying to identify the PKC-responsive elements.

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## INDUCTION OF SPI-1 GENE EXPRESSION BY INTERFERON IN K562 HUMAN LEUKEMIA CELLS

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The *spi*-1 proto-oncogene is transcriptionally deregulated in the murine erythroleukemia induced by the Friend retrovirus. It encodes the transcription factor PU.1, a member of the Ets family expressed in all hematopoietic cells except in T cell lineage. To determine if Spi-1/PU.1 is involved in the control of proliferation/differentiation of human myeloid cells, we analyzed its expression in K562 cells. K562 cell line is an attractive model as the cells can be chemically-induced to differentiate *in vitro* into two different lineages. 1-ß-D-arabinofuranosylcytosine (Ara-C, 1 µM) induces the irreversible growth arrest of the K562 cells and their differentiation in the erythrocytic pathway while the phorbol ester TPA (10 nM) induces their differentiation in the myelomonocytic pathway. Furthermore, treatment of K562 cells with the potent biological response modifiers interferon α-(2000 U/ml), ß (1000 U/ml) or γ (500 U/ml) results in inhibition of K562 cells proliferation without differentiation. *Spi*-1 expression in K562 cells was low in comparison to other human cell lines tested, as shown by Northern analysis, and the steady-state levels did not significatively change during induction of erythrocytic or myelomonocytic differentiation pathways. In contrast, treatment of K562 with interferon-α resulted in a strong increase of *spi*-1 mRNA after two days of induction. This effect was reversible since *spi*-1 expression returned to control levels after removal of IFN from the culture medium. Similar *spi*-1 up-regulation was observed in cells treated with ß and γ IFNs. Interestingly, the *spi*-1 up-regulation was inhibited when K562 cells were differentiated towards the erythrocytic pathway prior to IFN addition.

The above results were confirmed by studying the DNA binding activity of Spi-1/PU.1 in nuclear extracts from K562 cells by electrophoretic mobility shift assays. A large increase of specific Spi 1-DNA complexes were detected in extracts from cells induced with IFN-α up to 4 days in comparison to uninduced cells or to cells differentiated prior to IFN addition. Experiments to investigate the regulatory effect of interferons on *spi*-1 gene expression and its relation to proliferation and differentiation of K562 cells are in progress.

# Differences between embryonic/fetal and adult macrophages in mouse and chicken

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To investigate early myeloid development we established an in vitro differentiation (IVD) system for macrophages based on pluripotent mouse embryonic stem (ES) cells. As a molecular marker for macrophage differentiation processes we introduced the chicken lysoyzyme gene into the ES cell IVD system. The chicken lysozyme gene is known to be myeloid specifically expressed and to be activated gradually during macrophage development. The 20 kb gene domain has been well characterized and the functions of several of its cis-regulatory elements as well as different trans-acting factors involved in chicken lysozyme gene regulation are known. The complete chicken lysozyme gene domain has been shown to confer position-independent, cell type specific and high level expression of the gene in macrophages of transgenic mice.
In the study presented here ES cells were stably transfected with the chicken lysozyme gene locus. Cells from five independent clones were induced to differentiate into macrophages and expression of the transgene was analyzed. Chicken lysozyme gene expression within the ES cell IVD system was, as in transgenic mice, macrophage specific and independent of its position in the host genome. However, the expression level per gene copy, although constant amongst the 5 clones, was significantly lower than in macrophages from adult transgenic mice carrying the identical construct. Macrophages derived from places of embryonic/fetal hematopoiesis i.e. yolk sac, fetal liver and newborn liver also showed a low transgene expression level comparable to that of ES cell derived macrophages, whereas all macrophages derived from places of adult hematopoiesis, i.e. peritoneum, bone marrow, spleen and newborn spleen showed a constant high transgene expression level. A similar pattern was found when the endogenous chicken lysozyme gene was examined in chicken macrophages: retrovirally (MC29) transformed adult chicken macrophages express the gene at a high level, but MC29 transformed macrophages derived from embryonic chicken blastoderm possess only little lysozyme RNA. From these results we conclude that like in the erythroid and the lymphoid lineages also in the myeloid lineage differences between embryonic/fetal and adult macrophages exist and that the macrophages developing from ES cells in culture resemble the embryonic/fetal type of macrophage. Experiments investigating the underlying differences in transcription factor equipment and/or activity between the different cell types are under way.

**CYTOPLASMIC EXPRESSION OF BCL-2 PROTEIN IN ACUTE MYELOID LEUKEMIAS (AML)**

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We are assaying the expression of cytoplasmic bcl-2 protein (bcl-2) in patients diagnosed of AML, trying to evaluate its prognostic significance. So far bcl-2 has been assayed in 20 patients diagnosed of AML within the last 2 years. Preliminary results are shown.

**PATIENTS AND METHODS.** Patients were 9 female and 11 male, mean age 45 years (range 14-80). Typification of the leukemia was made by means of conventional morphological and cytochemical methods as well as immunocytochemistry, flow cytometry and karyotypic analysis with GTG banding; bcl-2 in the leukemic cells was assayed only by immunocytochemistry (Streptavidin-Biotin-Alkaline Phosphatase). Patients were arbitrarily clasified in 4 groups according to the percentage of cells expressing bcl-2: I, "negative", with no positive cells (6 patients); II, <20% positive cells (5 pts); III, 20-59 % positive cells (4 pts); IV, ≥60% positive cells (5 pts).

**RESULTS.** FAB subtypes of patients included in the different groups were as follows: Group I, 5 M2, 1 M4; Group II, 1 M1, 1 M3, 1 M4, 1 M5, 1 M6; Group III, 1 M0, 2 M1, 1 M2; Group IV, 2 M1, 1 M2, 1 M3, 1 M4. CD34 expression occurred in all 6 patients of Group I and in 5/14 (36 %) bcl-2 positive patients (Groups II-IV). Co-expression of lymphoid markers and bcl-2 was observed in 4/6 (67 %) of Group I (T or B markers) and in 6/14 (43 %) of Groups II-IV (only T markers). Metaphases for karyotypic analysis were obtained in only 10/20 patients at diagnosis; no evaluable metaphases were obtained from the 6 of Group I, 3 of Group II and 1 of Group IV. Karyotypic abnormalities were observed in 6: trisomy-8 in 2 and t(15;17) in 1 of Group IV; monosomy-21 in 1 and monosomy-10 in 1 of Group III; 5q- in 1 of Group II.

No treatment was given to 4 patients because of their inadequate clinical conditions. The only 3 of the 16 who were treated who did not achieve complete remission (CR) were bcl-2 positive: 2 of Group II and 1 of Group IV. Of the 13 who achieved CR, 4 were bcl-2 negative and 9 bcl-2 positive; 1 of these received an allogeneic Bone Marrow Transplant (BMT) and 12 have entered programs of intensive post-remission chemotherapy that, up to the date of this writting, only in 1 has already included autologous BMT; 11/13 remain in 1st CR, 2 to 16 months from diagnosis. AML has relapsed in 2, after 12 and 15 months from CR: one that initially had 25 % bcl-2 positive leukemic cells, showed 100 % positivity at relapse; the other was initially negative and showed 30 % positive leukemic cells at relapse.

**COMMENTS:** Cytoplasmic expression of bcl-2 has been observed in all FAB subtypes of AML, independently of CD34 or lymphoid markers expression. Karyotypic abnormalities of poor prognosis significance have been observed in bcl-2 positive patients. Both patients refractory to treatment were bcl-2 positive and both the 2 observed relapses showed bcl-2 expression, increased or anew. These preliminary data are consistent with a possible unfavorable prognostic significance of bcl-2 expression in AML cells.

## THE DNA-BINDING DOMAIN OF THE MYB ONCOPROTEIN - ROLE OF THE R2 REPEAT.

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The oncoprotein Myb is a sequence specific *trans*-activator, regulating genes important for proliferation and differentiation of hematopoietic cells (1). The DNA-binding region of the protein consists of three conserved repeats $R_1$, $R_2$ and $R_3$, where only $R_2$ and $R_3$ are required for sequence specific DNA binding. The $R_2$-domain plays a central role in the function of the protein. The v-*myb* protein of the AMV-virus contains three point mutations in $R_2$ that alters the differentiation of myelomonocytic cells transformed by the oncogene (2). $R_2$ also contains a highly conserved and oxidizable cystein (cys 43) which we believe to function as a molecular sensor of cellular redox signals (3). In addition the $R_2$ repeat contains a disordered region (4) that seems to undergo a conformational change upon binding to DNA, and this change in conformational seems to be controlled by the redox state of the cysteine in $R_2$ (3). The central role of the $R_2$ repeat in the function of Myb has been further studied along four lines of investigation: (I) We have performed domain swaps experiments replacing the $R_2$ repeat in the minimal DNA-binding domain of Myb by different Myb-homologous repeats from the yeast Bas1 protein (5). The sequence recognized by the fusion protein was identified using the "target detection assay" and showed that $R_3$ plays a dominant role in sequence recognition. (II) We have introduced a series of mutations in the region of $R_2$ where the AMV-specific point mutations are found, and have studied the mutant proteins with respect to DNA-binding properties and protein conformation. (III) We have established a yeast effector-reporter system that seems to be very sensitive to $R_2$-mutations. In particular, the system revealed a significant effect of the AMV-specific $R_2$-mutations. (IV) We have investigated whether the redox sensitive cystein in $R_2$ is a potential target for the thiol reactive signal molecule NO. NO-mediated nitrosylation of the cysteine in $R_2$ gives a protein inactive in sequence specific DNA-binding. In conclusion we believe that the Myb $R_2$-repeat could have regulatory roles beyond its function as an anchorage for $R_3$ as suggested by the NMR-structure of the $R_2R_3$-DNA complex (6).

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## TRANSCRIPTION FACTORS INVOLVED IN THE LEUKOCYTE-RESTRICTED AND THE REGULATED EXPRESSION OF LFA-1 AND p150,95 INTEGRINS

**Cristina López-Rodríguez, Arsenio Nueda and A. L. Corbí**

The ß2 or leukocyte-restricted Integrins, which include the LFA-1, Mac-1 and p150,95 (CD11a-c/CD18) heterodimers, play a key role in cell-cell interactions during immune and inflammatory reactions, as demonstrated by the existence of an immune disorder termed Leukocyte Adhesion Deficiency. LFA-1 is the only integrin expressed on all leukocyte lineages and is involved in the cellular adhesion events required for CTL and NK-mediated cytotoxicity, T and B lymphocyte responses, granulocyte and monocyte antibody-dependent cell cytotoxicity, and leukocyte extravasation into inflamed tissues. On the other hand, p150,95 is almost exclusively expressed on cells of the myeloid lineage and on activated B lymphocytes, and its expression is regulated during cell activation and differentiation by transcriptional mechanisms acting on the CD11c gene. The p150,95 integrin mediates CTL-mediated cytotoxicity, leukocyte adhesion to endothelium, and is a receptor for fibrinogen and the complement component iC3b. We have previously demonstrated that the proximal region of the CD11a and CD11c promoters are sufficient to direct the tissue-restricted and developmentally-regulated expression of LFA-1 and p150,95 (López-Cabrera et al., J. Biol. Chem. 268:1187,1993; Nueda et al., J. Biol. Chem. 268:19305, 1993).

Experiments designed to identify the DNA elements and transcription factors responsible for the unique pattern of p150,95 expression have demonstrated the presence of two Sp1-binding sites at -70 and -120 in the CD11c proximal promoter. Sp1 was demonstrated to transactivate the CD11c promoter in SL2 cells and mutations at the Sp1 proximal site (Sp1-70) greatly decreased the Sp1 transactivation capacity, whereas mutation of the distal Sp1 site (Sp1-120) had only a minor effect. More importantly, mutation of either Sp1 site led to a reduction in the basal CD11c promoter activity in myeloid U937 cells, while the opposite effect was observed in the CD11c-negative HeLa cell line, indicating the involvement of both Sp1 sites in the tissue-specificity of the CD11c expression, a fact further confirmed by *in vivo* footprinting. On the other hand, to identify transcription factors involved in the developmental regulation of the CD11c gene, we have used the U937 myelomonocytic cell line, whose PMA-driven monocytic differentiation induces the transcription of the CD11c gene and, consequently, the cell surface expression of p150,95. EMSAs demonstrated the presence of an AP-1-binding site within the CD11c gene promoter (AP1-60) and identified c-jun, junB, c-fos and Fra-1 as the members of the AP-1 family interacting with the AP1-60 site in differentiated U937 cells. The actual involvement of AP1-60 in DNA-protein interactions was also determined by means of *in vivo* footprinting experiments, and its functionality was demonstrated by transactivation of the CD11c proximal promoter by a c-jun expression vector in the AP-1-deficient F9 murine teratocarcinoma cell line. Site-directed mutagenesis of the AP1-60 site greatly inhibited the basal promoter activity as well as the induction of the CD11c promoter activity during the PMA-driven U937 cell differentiation. Additional DNA elements involved in the developmentally-regulated expression of CD11c (b-HLH and Myb consensus sequences) have been mapped by means of *in vivo* footprinting, functional analysis of serial 5'deletion mutants and the construction of U937 stable transfectants containing the CD11c promoter ligated to the luciferase gene reporter.

In summary, the AP-1 transcription factor complex plays an important role in both the basal and the developmentally regulated expression of the p150,95 leukocyte integrin, while the Sp1 transcription factor is involved in both the basal and the tissue-specific expression of the CD11c gene. The involvement of Sp1 and AP-1 in the transcription of the CD11c gene raises the possibility that integrin expression is controlled by the Retinoblastoma gene product and may facilitate altering leukocyte integrin expression by pharmacological means (e.g. glucocorticoids).

Additional structural and functional studies on the leukocyte integrin genes have also demonstrated the importance of an Octamer sequence in the transcription of the CD11c gene, and the relevance of the macrophage- and B cell-specific PU.1 transcription factor for the transcription of the CD11a and CD11c genes. The importance of these and other transcription factors (e.g. GABP) in the control of integrin expression will be discussed.

## MDM2, p21/WAF1 & P53 EXPRESSION. A comparative study in normal and mytogenically stimulated peripheral blood lymphocytes (PBL's), reactive lymphoid tissue and high grade non-Hodgkin's lymphomas (NHL's).

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MDM2, p21/WAF1 and p53 protein expression along the cell cycle was determined by flow cytometry on phytohaemagglutinin (PHA)–stimulated PBL's, simultaneously with S–fraction determination. MDM2, p21/WAF1 and p53 expression increased from day 0 to day 3 poststimulation. This increase occur in parallel to the number of cells in S phase. These results were confirmed by western blot.

In reactive lymphoid tissue (tonsils), MDM2, p21/WAF1 and p53 protein expression was detected by inmunohistochemistry, with a previous step of microwave irradiation. The three proteins were observed in isolated germinal center cells, some suprabasal epithelial cells and in ocasional endothelial nuclei. The number of positive cells was greater, and staining intensity was stronger for p53 than for p21/WAF1 and MDM2.

Levels of these three proteins were determined in 14 HG NHL's with the p53 gene status known from a previous sequence study of the conserved region (exons 4 to 9), 4 of which had point mutations. One of these cases had a point mutation leading to a stop codon, with neither p53 nor MDM2 and p21/WAF1 expression. The other 3 cases with p53 point mutations overexpressed p53, with either absent or very weak MDM2 and p21/WAF1 expression.

In the remaining 10 cases with germline p53 configuration, 8 cases displayed similar p53 p21/WAF1 and MDM2 expression. Two cases overexpressed p53 with low or absent MDM2 and p21/WAF1 expression. These last two cases could be candidates for p53 mutations outside the conserved region.

MDM2, p21/WAF1 and p53 expression in PHA activated lymphocytes and in reactive lymphoid tissue, is probably the expression of biological signals regulating cell proliferation.

HG-NHL cases with p53 mutation had very low or undetectable levels of MDM2, and p21/WAF1 protein. This supports that the expression of these proteins is induced by wild type p53 in these cases.

These findings suggests that MDM2, p21/WAF1, and p53 may play a role in the regulation of growth control in lymphocytes and reactive lymphoid tissue, and suggest that their deregulation may be implicated in the development of HG-NHL's.

# List of Invited Speakers

| | |
|---|---|
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*The lectures summarized in this publication were presented by their authors at a workshop held on the 24th through the 26th of April, 1995, at the Instituto Juan March.*

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