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# Workshop on Molecular Diagnosis of Cancer

Organized by
M. Perucho and P. García Barreno

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249 Workshop on Molecular Diagnosis of Cancer

Fundación Juan March

Serie Universitaria

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# I N D E X

**PROGRAM: WORKSHOP ON "MOLECULAR DIAGNOSIS OF CANCER"**

November 27

Morning: **Mariano Barbacid** (Chairman): *RAS* ONCOGENES

1.- **Frank McCormick** - The role of GAP in signal transduction
2.- **Angel Pellicer** - *Ras* oncogene activation in human skin tumors and in radiation and chemically induced animal tumors.
3.- **Johannes Bos** - The role of *ras* oncogenes in human cancer
4.- **Manuel Perucho** - Expression and activation of the c-K-*ras* gene in human carcinomas

Afternoon: **Frank McCormick** (Chairman): TUMOR SUPPRESSOR GENES

1.- **Robert Weinberg** - The retinoblastoma gene
2.- **Edward Harlow** - Cellular targets for transformation by DNA tumor viruses
3.- **Eric Fearon** - Genetic changes in colo-rectal tumor progression
4.- **Manfred Schwab** - Molecular biology of neuroblastoma

November 28

Morning: **Dennis Slamon** (Chairman): NUCLEAR ONCOGENES, DNA VIRUSES

1.- **Fred Alt** - Transgenic and homologous recombination models to study *myc* gene function
2.- **Riccardo DallaFavera** - Oncogenes in lymphoid malignancies
3.- **Premkumar Reddy** - Transcriptional regulation and function of *myb* oncogene
4.- **Ethel-Michele de Villiers** - Human papillomaviruses in human anogenital cancer

Afternoon: **Manuel Perucho** (Chairman): SHORT ORAL PRESENTATIONS

1.- **Juan C. Lacal** - *Ras* proteins as potential regulators of protein kinase C function in fibroblasts and neuronal cells
2.- **Robert Slebos** - Detection of mutations in codon 12 of the K-*ras* oncogene DNA and RNA from human non-small cell lung carcinomas
3.- **Miguel Quintanilla** - Cytokeratin expression of mouse epidermal keratinocytes during the process of neoplastic conversion
4.- **Demetrios Spandidos** - Ras oncogenes in cell transformation and human cancer
5.- **Jun Yokota** - Multiple genetic alterations in small-cell lung carcinoma
6.- **Evelyn May** - Human breast cancer: identification of populations with high risk of relapse in relation to estrogen status and HER-2 overexpression
7.- **Massimo Tommasino** - Expression of HPV16 transforming protein E7 in fission yeast *S. pombe*
8.- **Federico Garrido** - MHC antigen expression and oncogene activation on tumors

November 29 (Public Session)

Morning: **Pedro G. Barreno** (Chairman): GENE AMPLIFICATION, TRANSLOCATIONS

1.- **Dennis Slamon** - Alterations of the *erb*2/neu protooncogene in human breast and ovarian cancers
2.- **Igor Roninson** - Multidrug resistance
3.- **John Groffen** - Diagnosis and implications of Ph-positive leukemia
4.- **Mariano Barbacid** - Workshop summary

# INTRODUCTION

**M. PERUCHO**
California Institute of Biological Research, La Jolla (USA)

## INTRODUCTION

It is becoming a paradigm in cancer research that combinations of genetic alterations resulting in the activation of cellular oncogenes and in the inactivation of tumor suppressor genes are the underlying causes of malignant transformation and tumor progression. Oncogenes are the activated form of certain cellular genes (protooncogenes) which are involved in cell growth and/or differentiation. Tumor suppressor genes or suppressors of cell growth are those genes which play a negative regulatory role in the same process(es).

Each of the steps that comprise the process of signal transduction leading to cell replication are potential targets for oncogene activation and presumably also for the inactivation of tumor suppressor genes. Thus, the extracellular, environmental factors (growth factors, hormones, etc.) tell the cell to divide or differentiate, by interacting with their corresponding receptors located in the cellular membrane. The signal is then transmitted through other membrane-bound, regulatory intermediate proteins, by a process that involves cycles of protein phosphorylation and dephosphorylation by the action of protein kinases and phosphatases. The change in intracellular environment resulting from these processes is finally conveyed to the nucleus where, presumably by the action of nuclear proteins (transcriptional factors and regulators), the order to divide or differentiate is finally executed.

This complex process, which has been finely tuned through billions of years of evolution by natural selection, is threatened by accumulative genetic damage resulting from spontaneous copying errors during DNA replication or from the action of external toxic agents. Despite the fail-safe mechanisms provided by evolution, the equilibrium between growth stimulation and repression is then eventually broken (generally after the organism reproductive period), and malignant transformation occurs. The unraveling of the complex interplay between oncogene activation and tumor suppressor gene inactivation is therefore one of the most intense areas of modern cancer research. Gene amplification, chromosomal translocations, genetic deletions, and single point mutations mediate oncogene activation. Point mutations together with small or large chromosomal deletions are responsible for inactivation of tumor suppressor genes.

The development of sophisticated recombinant DNA technologies able to detect the variety of genetic alterations associated with these two opposite phenomena has resulted necessarily in applications for cancer diagnosis at the molecular genetic level. The diagnostic classification of neoplasias based on the presence or absence of these tumor-specific genetic alterations has provided, in some cases, an additional value for cancer prognosis. It is also obvious that this molecular diagnosis of human tumors should provide critical information after the predictable development of new therapeutic modalities, directed to altered oncogene products.

With these considerations in mind, it was thought that it would be timely and appropriate to organize a workshop to discuss the recent advances in basic research on oncogenes and tumor suppressor genes and its application for cancer diagnosis and prognosis. Under the auspices of the Fundación Juan March, a workshop was held in Madrid in November of 1989 with the participation of 16 speakers and about 30 invited participants, most of them Spanish.

# RAS ONCOGENES

F. McCORMICK
A. PELLICER
J.L. BOS
M. PERUCHO

**PROPERTIES OF ras p21 GTPase ACTIVATING PROTEIN (GAP)**

**Frank McCormick**
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GAP is a 120 kd protein found in all mammalian cells. It acts catalytically to convert *ras* p21.GTP to *ras* p21.GDP, thus inactivating the p21 protein. Mutations that convert *ras* proto-oncogenes into oncogenes (at positions 12, 13, 61, etc.) allow p21 to escape from GAP action and thus exist in the GTP-bound state constitutively.

We have expressed human GAP cDNAs in insect cells using baculovirus vectors, and have been able to purify GAP from these cells to 95% purity. This recombinant GAP is at least 10-fold more active than GAP purified from mammalian cells, possibly because of different post-translational modifications. To determine whether GAP is a substrate for phosphorylation by tyrosine kinases, we co-expressed GAP with v-src, c-src, c-abl (types I and IV), P210 and P185 bcr/abl proteins, EGF-R or insulin receptor. In most cases tyrosine phosphorylation was observed. c-Abl proteins, however, did not phosphorylate GAP. These proteins (characterized in collaboration with Owen Witte and Ann-Marie Pendergast) have tyrosine kinase activity when expressed on their own in insect cells, but have reduced activity when co-expressed with GAP. Furthermore, GAP forms stable complexes with c-abl proteins, and with P210 and P185 bcr/abl proteins.

In mammalian cells, GAP is a major substrate for phosphorylation by tyrosine kinase oncogenes (Ellis, Moran, McCormick and Pawson, in the press). Phosphorylated GAP associates with two other tyrosine kinase substrates, designated P210 and P60. The identity of these proteins is unknown. Phosphorylation of GAP by tyrosine kinases decreases its ability to promote hydrolysis of GTP bound to p21. The significance of this will be discussed. Phosphorylated GAP also binds to PDGF-receptor, *in vivo* and *in vitro* (Kaplan, Morrison, Wong, McCormick and Williams, submitted). However, GAP does not bind to PDGF-receptor mutants that lack the kinase-insert domain, nor to PDGF-receptor in *ras*-transformed cells. These results indicate that binding of GAP to PDGF-receptor is a necessary part of mitogenic signalling.

## RAS ONCOGENE ACTIVATION IN HUMAN SKIN TUMORS AND IN RADIATION AND CHEMICALLY INDUCED ANIMAL TUMORS

**A. Pellicer**

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*Ras* oncogenes have been found to be activated in many types of tumors being the most prevalently activated type of oncogene compiling all types of human cancers.

The search for activated *ras* oncogenes has been primarily directed towards the screening of malignant tumors due to the clinical impact of these entities. We wanted to analyze the presence of *ras*-activated oncogenes in benign tumors to determine if *ras* activation was a feature of malignancy or, on the other hand, it could be present before the invasive features appeared.

We chose a tumor system in which the benign tumors also spontaneously regress. The same type of tumor that occurs in humans can be induced in an animal model system to facilitate its study and molecular dissection. The tumor is the keratoacanthoma (KA) which develops in the skin, and we induce in rabbits by painting the inner face of the ear with DMBA. Both in human and rabbits the tumors grow for a few weeks and subsequently regress.

The activation of *ras* oncogenes has been investigated by the classical methods of NIH 3T3 focus formation, nude mice tumorigenesis after cotransfection with a dominant marker and PCR amplification followed by oligonucleotide mismatch hybridization and direct DNA sequencing.

The rabbit tumors were more amenable to the biological assays due to the amounts of material that could be obtained. The results obtained with these assays indicated that a substantial percentage of the tumors (65%) contained activated H-*ras*. When these tumors were analyzed by the more sensitive techniques that included PCR as an initiating step, the frequency of activation of the H-*ras* oncogene increased to more than 90%. The analysis of the human samples was for most cases through the amplification of material from formalin-fixed, paraffin-embedded tissue that was obtained from the dermatopathology archives. Here, the activation of H-*ras* was in 30% of the samples which is a significant proportion taking in account that this is a benign and self-regressing tumor.

The analysis of the specific mutations detected in the tumors was the following: In the rabbit system over 90% of the tumors contained an A:T to T:A transversion in the second base of codon 61. In humans, the mutations were present in several positions but the most frequent was also the mutation detected in the rabbit tumors, suggesting either similar etiology or an unusual potency of that particular mutation in the skin that allows a more frequent selection. In several of the human tumors, the particular mutation was confirmed by sequencing the PCR-amplified material from the paraffin sections, indicating that this approach is feasible in a number of cases and with implications for the analysis of human samples that exist in pathology archives.

We decided next to compare the frequency of H-*ras* activation in the KA with the squamous cell carcinoma (SCC), the malignant tumor that shows more histopathological similarities with the KA. The result was that only 13% of the human SCCs analyzed contained H-*ras*-activating mutations, suggesting that in this system, *ras* mutations do not seem to be providing an advantage for the malignant phenotype. The analysis of SCCs that appear in the rabbit, albeit in a lower proportion than KAs, indicates that the frequency of the usual activating mutation in KAs is low, again suggesting that this *ras*-activating mutation might be a burden for the development of the malignancy. This is additionally supported by the fact that here the same agent is the cause of both KAs and SCCs. An alternative explanation in which both types of tumors develop using completely different tumorigenic pathways is also possible.

These studies indicate that the screening of selected human tumors for *ras* mutations can provide interesting clues for the role of this oncogene family in tumorigenesis.

## THE ROLE OF MUTATED RAS GENES IN HUMAN MALIGNANCIES

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The presence of activated RAS genes in various human malignancies was determined. We found mutated RAS genes in colon carcinomas (50%, mostly KRAS), lung adenocarcinomas (30%, mostly KRAS), acute myeloid leukemias (30%, mostly NRAS), seminomas (20%), pancreatic adenocarcinomas (85%, KRAS) and myelodysplastic syndrome (30%). No RAS gene mutations were found in cervical cancer, ovary tumors, glioblastomas, CML and follicular cell lymphomas. These results demonstrate that activated RAS genes do occur rather frequently in certain human malignancies, whereas they are absent in others. Analysis of premalignant precursor tissues such as colon adenomas and myelodysplasia indicates that the mutational event can occur relatively early but also late in the course of tumorigenesis. Comparing the presence of a mutated RAS gene and a variety of parameters does not reveal clear correlations with clinical or histopathological features of a tumor, but does reveal the involvement of mutagenic agents like tobacco smoke and UV in the induction of the RAS mutation.

In an effort to understand the function of Ras proteins in signal transduction, we have investigated rat-1 cells transfected with normal RAS genes. We observed that these cells exhibit a transformed phenotype which is dependent on the addition of serum. Subsequently, we discovered that in these cells insulin/IGF-I can replace serum in the induction of DNA synthesis, whereas the parental rat-1 cells do not respond to insulin/IGF-I. To further investigate the hypothesis that insulin/IGF-I might be ligands that can activate Ras proteins, we analyzed a variety of genes that are induced upon growth factor stimulation for their response on insulin/IGF-I. We found that, for instance, the FOS gene is not inducible by insulin/IGF-I in the parental rat-1 cells and in mutant RAS transformed cells, but strongly inducible in cells expressing the transfected normal RAS gene. These results suggest that Ras proteins are functionally linked to the insulin/IGF-I signal transduction pathway.

**EXPRESSION AND ACTIVATION OF THE C-K-RAS GENE IN HUMAN CARCINOMAS**

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The presence of point mutations at the first coding exon of the c-K-*ras* gene and the relative steady-state levels of mutant and normal c-K-*ras* transcripts were simultaneously analyzed by the RNAse A mismatch cleavage method in a panel of more than 300 primary human tumors obtained after surgery with curative or palliative intent. Point mutations were also detected in a series of more than 100 specimens obtained as formalin-fixed, paraffin blocks collected from surgical resections, biopsies and autopsies, by the RNAse A mismatch cleavge method after *in vitro* amplification by the Polymerase Chain Reaction (PCR) of c-K-*ras* sequences. The mutations were characterized in many of these tumors by hybridization with allelic-specific oligonucleotides, cloning and sequencing of the PCR products and/or restriction fragment length polymorphism (RFLP) either naturally created by the mutations or artificially introduced by the use of mutant PCR primers. We have also shown that this methodology offers a complementary approach for the molecular diagnosis of human cancer.

Mutant c-K-*ras* genes were found in 80% of pancreatic adenocarcinomas, in 40% of colorectal adenomas and carcinomas, in 25% of adenocarcinomas of the lung and in one-third of carcinomas of the extrahepatic bile duct, cholangiocarcinomas and ampullary carcinomas. The mutation frequency was lower (5-10%) in renal carcinomas and squamous cell carcinomas of the lung or absent in carcinomas of the breast, stomach, prostate, uterus and ovary, and melanomas, among others.

The distribution of the mutations was similar in tumors of the colon and rectum and of the exocrine pancreas, with the most prevalent mutation being the G to A transition at the second position of codon 12 resulting in the replacement of glycine for aspartic acid (42% and 45%, respectively).

Our analysis of about 250 colorectal tumors has revealed a series of statistically significant correlations between c-K-*ras* mutations and some clinical, histopathological and ethnic parameters. Tumors with mutations at codon 13 were older than those with mutations at codon 12, and codon 13 mutations were also more prevalent in adenomas than in carcinomas. Mutant c-K-*ras* genes were found in 8 of 8 villous adenomas and in 7 of 8 carcinomas originating in

villous adenomas and c-K-*ras* activation was associated prevalently with carcinomas of well differentiated phenotype.

The incidence of c-K-*ras* mutations also show a slight but significant steady increase in tumors at more advanced stages of progression, and the ratio of mutant versus normal allele expression also increases in parallel with tumor progression. We have also shown that although amplification and overexpression of the mutant allele relative to the normal is a frequent finding, malignant cells can be functionally heterozygous for the mutant c-K-*ras* allele.

These results support the concept that mutant *ras* gene products play an important role in human carcinogenesis, contributing in a dominant but dose-dependent manner to tumor progression, including the metastatic process. Our results also are consistent with the hypothesis that the biochemical differences of *ras* proteins containing distinct amino-acid substitutions might be reflected in the phenotypic as well as the clinical characteristics of the corresponding tumors.

# TUMOR SUPPRESSOR GENES

R.A. WEINBERG
E. HARLOW
E.R. FEARON
M. SCHWAB

## THE RETINOBLASTOMA GENE

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Cytogenetic analysis of retinoblastoma tumors has revealed interstitial deletions involving band 13q14. This has implicated a gene, termed Rb, the deletion of which triggers retinoblastomas in children up to the age of 5. This gene has now been isolated as molecular clones. It encompasses ca. 190 kilobases of DNA on chromosome 13 and encodes a 105 kilodalton nuclear phosphoprotein. Antisera generated against this protein have shown it to be expressed in a wide variety of tissues including the normal fetal retina. Significantly, this protein was found to be absent from 18 out of 18 retinoblastoma tumor samples and cell lines examined. Moreover, use of such antisera reveals that the protein is either absent or present in altered form in virtually all small cell lung carcinomas and in about one-third of bladder carcinomas. Southern blot analysis of sarcomas has also shown that the gene is frequently altered in sarcomas as well.

The Rb-encoded protein, p105, is found exclusively in the nucleus. In cells infected by DNA tumor viruses, the protein is often found in a form that is complexed with virus-encoded oncoproteins. This was initially discovered with the adenovirus E1A oncoprotein and has since been extended to the products of the SV40 large T oncogene and the human papillomavirus E7 oncogene. These oncogenes, all of which immortalize embryo cells and collaborate with *ras* oncogenes in transformation, may act by inactivating p105-Rb and, in so doing, mimic the state seen in retinoblastoma cells that have lost this protein through inactivation of their Rb genes.

**CELLULAR TARGETS FOR TRANSFORMATION BY DNA TUMOR VIRUSES**

Nicholas Dyson, Karen Buchkovich, Qianjin Hu, and **Ed Harlow**
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Tumor suppressor genes are genetic loci whose loss has been associated with tumor development. Because the inactivation of both alleles of these genes is a key feature in the genesis of certain tumors, it has been postulated that the protein products of tumor suppressor genes function in the negative regulation of cell proliferation. Tumor suppressor genes have been identified by genetic analysis either as loci associated with an inherited predisposition to certain tumors or by karyotypic studies that have localized putative tumor suppressor genes to loci that show reduction to homozygosity or loss of heterozygosity during tumor development. Second, recent work from a number of laboratories has shown that the protein products of tumor suppressor genes often form protein/protein complexes with the transforming proteins of small DNA tumor viruses. The retinoblastoma gene, RB-1, is one of the best studied examples of the tumor suppressor genes. It was originally identified and cloned through its association with childhood retinoblastoma. However, it is also a key target for transformation by the oncogenes of several small DNA tumor viruses. The E1A proteins of adenovirus, the large T antigens of polyomaviruses, and E7 proteins of papillomaviruses all bind to p105-RB. Genetic studies of all three viruses have shown that any mutation that destroys binding to p105-RB also destroys the ability of these proteins to transform cells, suggesting that interaction with the RB gene product is a key event in viral transformation. In addition to interacting with p105-RB, the adenovirus E1A proteins and the polyomavirus large T antigens also bind to other cellular proteins. One of these, a protein with a molecular weight of 107,000 daltons, 107K, binds to E1A and large T at the same amino acid region as p105-RB, suggesting that the 107K and p105-RB proteins may have structural similarities. These observations and other comparisons raise the question whether 107K may be functionally related to p105-RB. If so, the 107K protein may be another example of a product of a tumor suppressor gene interacting with the transforming proteins of small DNA tumor viruses.

**IDENTIFICATION OF A CHROMOSOME 18q GENE WHICH IS ALTERED IN COLORECTAL CANCERS**

**Eric R. Fearon** and Bert Vogelstein
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The presence of chromosomal losses in human tumors has been suggested to provide evidence that the affected regions contain tumor suppressor genes. Allelic deletions involving chromosome 18q are present in over 70% of colorectal cancers, and we sought to identify a candidate suppressor gene on this chromosomal arm. A contiguous stretch of DNA comprising 370 kilobase pairs (kb) was cloned from a region of chromosome 18q suspected to reside near this gene. Human-rat sequence identities were used to identify potential exons in the 370 kb region; the expression of potential exons was assessed using an "exon-connection" strategy based on the polymerase chain reaction. Using expressed exons as probes for cDNA screening, clones were obtained which encoded a portion of a gene termed DCC; this cDNA was encoded by at least 8 exons within the 370 kb genomic region. The DCC gene was expressed in almost all normal tissues tested, including colonic mucosa, but DCC expression was greatly reduced or absent in over 85% of colorectal carcinoma cell lines studied. Such reduced expression in tumor cells is consistent with a suppressive function for the gene. In addition, somatic mutations of the DCC gene were observed in 12 of 95 colorectal carcinomas. These mutations included a homozygous deletion of the 5′ end of the gene, a point mutation within one of the introns, and 10 examples of DNA insertions within a 0.17 kb fragment immediately downstream of one of the exons. These findings suggest that the DCC gene should be considered as a candidate colorectal tumor suppressor.

**AMPLIFICATION OF MYCN IS PROGNOSTIC FOR POOR OUTCOME OF PATIENTS WITH NEUROBLASTOMA**

**Manfred Schwab**

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Neuroblastoma is a tumor of the sympathetic nerve system afflicting young children. Cells of neuroblastomas often contain conspicuous chromosomal abnormalities referred to as "double minutes" (DMs) and "homogeneously staining chromosomal regions" (HSRs) which are diagnostic for the presence of amplified DNA. All neuroblastomas showing DMs or HSRs were found to carry amplification of the gene MYCN, a kin of the MYC protooncogene. The initial surveys suggested that MYCN amplification was specific for neuroblastoma. It turned out that MYCN amplification can be seen in small cell lung cancer, retinoblastoma and astrocytoma, although at much lower incidence. As a common feature, all these tumors have neural qualities. Until now, MYCN has been the only gene, however, found amplified in neuroblastomas.

In this context, it should also be mentioned that neuroblastomas have lost genetic material from chromosome 1p at high incidence, and it has been suggested that the two genetic events of MYCN amplification and 1p deletion are related. In a recent study, we have employed molecular probes generated by microdissection and microcloning of the 1p36 region to identify loss of genetic information from 1p36.1-2 in at least 9 out of 10 neuroblastomas. Only two of these tumors had MYCN amplification. This result makes a relation between amplification of MYCN and deletion of 1p DNA unlikely.

The oncogenic potential of enhanced expression of MYCN as the consequence of amplification has been addressed in various experimental systems. Enhanced expression, resulting after introduction of a MYCN expression vector, can assist mutationally activated HRAS in tumorigenic conversion of primary rat embryo cells, convert established cells of the rat and of humans to tumorigenicity, and rescue primary rat embryo cells from senescence. These results clearly attest to the capacity of high MYCN expression to modulate the growth of cells, and it appears reasonable, therefore, to suggest that enhanced expression consequent to amplification contributes to tumorigenesis. The available evidence suggests that the nucleotide sequence of MYCN in neuroblastoma cells is unaltered compared to that of normal cells. Consistent with this result, the biological

activities of MYCN derived from normal or from neuroblastoma cells have not been found to differ.

An important prognostic variable for patients with neuroblastoma is the clinical stage. Patients with disease stage I and II have good prognosis with 75% to 90% 2-year disease-free survival, while patients with stage III and IV have a poor prognosis with 10% to 30% 2-year survival. Surveys of over 250 neuroblastomas revealed that a strong correlation exists between MYCN amplification and stage III and IV. Few patients with stage II tumors carrying amplification were identified. In all these instances, the tumor turned out to be fatal.

A peculiar stage IV characterized by frequent apparently spontaneous regression and good prognosis rarely shows amplification (7%). Three patients with MYCN amplification have been published. In all cases, the tumor progressed later. These observations make it advisable to subject patients with low stage tumor carrying amplification to more sophisticated therapeutic regimen.

A significant correlation between poor prognosis and MYCN amplification has also emerged when patients over 1 year of age were compared with patients under 1 year. The prognosis of patients above 1 year mainly diagnosed with stage III or IV tumors is particularly poor, and metastases occur preferably in bone, orbita and distant lymph nodes. More than 50% of the patients over 1 year carried amplification of MYCN, while amplification was rarely seen in patients below 1 year of age.

Altogether, MYCN amplification is associated with a higher malignant phenotype of neuroblastoma and is a good indicator for poor prognosis in patients with low stage disease. Amplification of MYCN represents the first instance in which the alterations of a cellular oncogene turned out to be of clinical significance.

# NUCLEAR ONCOGENES, DNA VIRUSES

F.W. ALT
R. DALLA FAVERA
E.P. REDDY
E.-M. DE VILLIERS

**ACTIVITY OF THE MYC FAMILY OF NUCLEAR ONCOGENES IN NORMAL AND MALIGNANT CELLS**

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The *myc* family of nuclear oncogenes is a dispersed multi-gene family that consists of three well-defined members: c-*myc*, N-*myc*, and L-*myc*. The c-*myc* gene is expressed in most dividing cells, and its deregulated expression by a variety of different mechanisms has been implicated in many different types of tumors. The N-*myc* and L-*myc* genes are expressed in a much more restricted set of normal cells, usually only in the very early stages of many different cell lineages. Correspondingly, deregulated expression of these genes has been implicated in a very limited set of naturally-occurring tumors (e.g., neuroblastoma and small cell lung carcinoma) and generally only by the mechanism of gene amplification. Amplification of the N-*myc* gene has been a clinically useful staging and prognostic indicator in neuroblastomas.

Unique structural aspects of individual *myc*-family genes and their distinct expression patterns during mammalian development suggests that their differential and/or combinatorial expression may play a role in differentiation of cells through particular lineages. We have focused on the potential function of differential *myc* gene expression during lymphocyte differentiation. Both N- and c-*myc* are expressed in precursor B and T lymphocytes but only c-*myc* is expressed after the cells acquire their antigen receptor and differentiate into mature B lymphocytes; L-*myc* is not expressed in known differentiation stages of the B cell lineage but it expressed in fetal thymus and certain immature T cell tumors. A special role of N-*myc* in precursor B lymphocytes is suggested by the fact that expression of N-*myc* but not c-*myc* is dramatically induced by treatment of normal precursor B cells with the pre-B growth factor IL-7.

To study the differential control and functions of *myc*-family genes, we have generated a variety of transgenic mouse lines that contain introduced *myc* genes expressed under the influence of their own regulatory elements or specifically targeted and deregulated within the lymphoid lineage. We have previously reported that transgenic mice carrying an N-*myc* gene deregulated by an associated immunoglobulin heavy chain enhancer element (E$\mu$-N-*myc*) is expressed

primarily in B lineage cells and leads to a predisposition to pre-B and B-cell malignancies in recipient mice. Molecular characterizations of B cell stage tumors in Eμ-N-*myc* mice demonstrate that these express a number of genes previously found to be expressed only at the pre-B stage including Ig VDJ recombinase activity. Spontaneous tumors arising as a result of the deregulated N-*myc* expression do not express the endogenous c-*myc* or N-*myc* genes; the lack of c-*myc* expression occurs at the transcriptional level and appears to be an indirect effect of high level N-*myc* expression.

In contrast to Eμ-N-*myc* mice, Eμ-L-*myc* transgenic mice show an unexpectedly high level of L-*myc* transgene expression in thymocytes and mature proliferating T cells as compared to B-lineage cells. Expansion of the thymic cortex and irregularly formed splenic follicles with expanded T cell areas are consistent histological abnormalities of the Eμ-L-myc animals. Eμ-N-*myc*/Eμ-L-*myc* double transgenic animals express N-*myc* primarily in B lineage cells and L-*myc* primarily in T lineage cells, despite the fact that both *myc* genes are inserted into identical immunoglobulin enhancer-containing constructs. These data suggest the existence of control elements within the L-*myc* gene that may act in concert with Eμ to provide preferential expression in T lineage cells. In correspondence to the Eμ-L-*myc* expression patterns, Eμ-L-*myc* mice show a predisposition to develop T cell lymphomas. In all but one of 10 characterized lymphoid malignancies in Eμ-L-*myc* mice, expression of the endogenous c-*myc* gene was down-regulated. As previously indicated in rat embryo fibroblast co-transformation assays, the deregulated L-*myc* gene appears to have a much weaker transforming activity than c- or N-*myc*. However, the absence of c- or N-*myc* expression in the tumors that arise indicate that L-*myc*, at least when expressed at high levels, can replace N- or c-*myc* potential functions with respect to the proliferation of these cells.

As an alternative model to examine N-*myc* function, we have disrupted the endogenous N-*myc* gene in multiple embryonic stem cell and pre-B cell lines by homologous recombination. We found that selection of these cell lines for expression of a promoterless construct containing murine N-*myc* genomic sequences fused to a gene encoding neomycin resistance allows highly efficient recovery of variants in which the endogenous N-*myc* gene is disrupted. The very high frequency of N-*myc* disruption by this method is being used in conjunction with secondary dominant disruption vectors to interrupt both allelic N-*myc* copies in such cell lines to study N-*myc* function. We have also used multiple disrupted ES clones for blastocyst injections, but, to date, resulting chimeric mice have

not carried the N-*myc* gene in their germline. In anticipation of the eventual generation of chimeras with germline N-*myc* disruptions and the possibility that such a mutation may be lethal in the homozygous state, we have also created a series of complementary transgenic mice that carry either the human or murine N-*myc* gene expressed from their own regulatory elements in normal or aberrant fashion. Comparison of expression patterns of the N-*myc* gene in transgenic mice as compared to transfected cell lines suggest that early developmental events may be necessary to establish ability for correct N-*myc* gene regulation at later developmental stages.

**ONCOGENES IN HUMAN B-CELL MALIGNANCIES**

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B-cell malignancies in humans represent a heterogeneous group of neoplasia characterized by phenotypically distinct neoplasia such as acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL) and multiple myeloma (MM). This heterogeneity most likely reflects the different stage of differentiation of the involved cell as well as the involvement of different pathogenetic mechanisms. In fact, specific tumor subtypes have been found associated with different types of oncogenes, although most tumor subtypes are also heterogeneous. The recapitulation of the pathogenetic steps involved in each type of malignancy will allow a better understanding of the biological action of oncogenes and the development of novel diagnostic methods based on the type of pathogenetic lesion.

Oncogenes that have been found consistently associated with different subtypes of B-cell malignancy include: i) H-, K-, and N-*ras*. Point mutations at specific codons (12, 13, 61) of these genes were detectable in ALL (18% of cases) and multiple myeloma (30%), while no *ras* mutations were found in CLL and NHL (1-2); ii) c-*myc*. Activation by chromosomal translocation of the c-*myc* oncogene are found in high-grade non-Hodgkin lymphoma (NHL), mainly Burkitt-type, in L3-type acute lymphoblastic leukemia (ALL) and in 70% of AIDS-associated non-Hodgkin lymphoma (AIDS-NHL) (3). These translocations are associated with structural alterations of the first exon of the c-*myc* gene and with specific alterations in the pattern of c-*myc* transcription (4); iii) *bcl*-2. Activation of the *bcl*-2 oncogene by the t(14;18) chromosomal translocation is detectable in 90% of follicular lymphoma and in 25% of diffuse lymphoma (5).

To gain insight into the role of these oncogenes in B-cell transformation, we studied their biological effects in an *in vitro* transformation assay in which Epstein-Barr virus (EBV)-immortalized B lymphoblastoid cells (LCL) are used as targets (6). In this assay, various oncogenes are introduced into LCL using plasmid or retroviral vectors and the cell phenotype is then analyzed for changes in transformation-related properties (serum-dependency, clonogenicity in semi-solid media and tumorigenicity in mice) as well as for changes in the expression of activation- or differentiation-associated markers detected by specific

monoclonal antibodies. In this assay, all four oncogenes induced malignant transformation, although at different degrees, but striking differences were detectable in the phenotype of the transformed LCL. In particular: i) H- and N-*ras* oncogenes induced malignant transformation and plasmacytoid differentiation leading to a phenotype analogous to MM cells (7); ii) the *bcl-2* oncogene induced a weakly transformed phenotype with no apparent changes in the differentiation stage, but was very effective in potentiating c-*myc*-induced transformation and tumorigenicity (8); iii) the c-*myc* oncogene-induced transformation characterized by the down-regulation of the adhesion-receptor LFA-1 and by the ability of escape autologous T-cell-mediated cytotoxicity, two features typical of Burkitt lymphoma cells.

We will discuss the implications of these findings for the role of the various oncogenes in different lymphoid malignancies as well as for the role of the corresponding proto-oncogenes in normal lymphoid development.

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**THE myb ONCOGENE: TRANSCRIPTION REGULATION AND TRANSLATION**

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The molecular mechanisms that modulate c-myb mRNA levels in hematopoietic cells appear to involve premature transcription arrest in the first intron of the gene. We have examined the DNA-protein interactions within this first intron of c-myb gene and identified a 1.0 kb region which could be responsible for the transcriptional regulation of this gene. By using mobility shift assay we show a direct correlation between the extent of sequence specific protein binding to some of the DNA fragments derived from intron I, with c-myb mRNA abundance in different cell types. During the DMSO induced differentiation of F-MEL cells there was a dramatic decrease in these nuclear factors as with the levels of c-myb mRNA. Nucleotide sequence analysis of this intronic region and DNase-1 foot-printing revealed the presence of cis-acting elements for the interaction of nuclear factors. We propose that binding of nuclear factors at the site of transcriptional pause could play an important role in the regulation of transcription of this gene.

c-Myb gene has previously been shown to code for a single protein species of about 75 Kd. However, accumulating evidence indicates that this gene could code for multiple mRNAs as a result of differential splicing. This was first detected in ABPL-2 tumor line and was later shown to occur in normal cells. To test if indeed these differentially spliced mRNA codes for new protein species that went hitherto undetected, a series of antibodies directed against specific domains of the myb protein, including the putative sequences that could be generated from the alternatively spliced mRNAS, were generated. Immunoprecipitation analysis using these antibodies show that both normal and tumor cell lines synthesize at least two myb proteins, one of 75 Kd and the other of 89 Kd.

**HUMAN PAPILLOMAVIRUSES IN HUMAN ANOGENITAL CANCER**

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The role of papillomaviruses in anogenital cancer is well established. Apart from the regular presence of viral DNA in cervical, vulvar, penile and perianal cancer biopsies, the viral DNA integrates in a specific pattern and specific viral genes (E6, E7) are regularly expressed within the proliferating cells. Recently, evidence has been accumulated pointing to a role of E6-E7 expression for the initiation and the maintenance of the proliferative phenotype. Our laboratory concentrated on the postulated role of host cell factors on papillomavirus expression in normal cells. Experimental studies point to a different regulation of viral DNA transcription in benign, when compared to malignant, cells. Besides anogenital cancer, additional human cancers (cancer of the skin, the oral mucosa, the larynx and the lung) are suspected to have a papillomavirus-linked etiology. Results on the demonstration of papillomavirus DNA in such cancers will be presented.

SHORT ORAL PRESENTATIONS

J.C. LACAL
R.J.C. SLEBOS
M. QUINTANILLA
A. CANO
D.A. SPANDIDOS
J. YOKOTA
E. MAY
M. TOMMASINO
F. GARRIDO

**RAS PROTEINS AS POTENTIAL REGULATORS OF PROTEIN KINASE C FUNCTION IN FIBROBLASTS AND NEURONAL CELLS**

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*Ras* proteins are essential elements for viability in a number of species (1,2). Their biochemical properties are similar to those of regulatory G proteins. Evidence has been provided which involves *ras*-p21 proteins in both proliferation and differentiation processes.

Protein kinase C (PKC) is a ubiquitous kinase whose activity depends on phospholipids and $Ca^{2+}$ (3). The enzyme can be activated by tumor promoters such as phorbol esters, which substitute the natural activator, 1,2-diacylglycerol (DAG). It is very well established that PKC is the endogenous receptor for phorbol esters. The enzyme is actually a family of isoenzymes with similar biochemical properties and different cell specificity. PKC has been related to a number of functions, including regulation of cell proliferation and neuronal differentiation, functions in which *ras*-p21 proteins are also thought to play an important role.

We have previously investigated the functional relationship between *ras* proteins and PKC in 3T3 cells, the model system for *ras*-induced malignant transformation of fibroblasts (4). Our results demonstrate the functional requirement of PKC for the mitogenic activity of H-*ras*-p21 protein, since removal of endogenous PKC by pretreatment of cells with PDBu for 48-72 hr inhibited more than 80% of the mitogenic activity of microinjected *ras*-p21 proteins. Co-microinjection of both *ras*-p21 and PKC restored the mitogenic activity induced by *ras*-p21.

Microinjection of transforming *ras* proteins into *Xenopus laevis* oocytes induce maturation (5). We have also demonstrated that *ras*-p21 proteins can induce a rapid generation of DAG when microinjected into *Xenopus* oocytes (6), and *ras*-transformed NIH 3T3 cells show constitutively elevated levels of DAG (7). We have not detected significant changes in the levels of inositol phosphates in *ras*-transformed cells. Instead, the levels of phosphocholine and phosphoethanolamine are significantly elevated, implying that *ras*-p21 proteins alter the metabolism of phospholipids other than polyphosphoinositides resulting the generation of elevated levels of DAG. Thus, *ras*-21 function might be related

to the regulation of PKC through the generation of DAG. Data supporting the functional relationship between *ras*-p21 and PKC will be presented.

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**DETECTION OF MUTATIONS IN CODON 12 OF K-ras ONCOGENE DNA AND mRNA FROM HUMAN NON-SMALL CELL LUNG CARCINOMAS**

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We have previously shown that mutations in codon 12 of K-*ras* are preferentially found in adenocarcinomas of the human lung, but not in any other lung tumor subtype (New Engl. J. Med. (1987), 317, 929). In none of these lung tumor DNAs a gene amplification of K-*ras* could be demonstrated (Br. J. Cancer (1988), 59, 76). All mutations were found in tumors from patients with a history of smoking, which suggests a direct relation of K-*ras* mutations with exposure to chemical carcinogens from tobacco smoke.

To be able to investigate the expression of both wild-type and mutated-K-*ras* in these tumors we have used the Polymerase Chain Reaction (PCR) on specifically primed cDNA to detect K-*ras* mRNAs.

RNA from 32 primary NSCLCs and from two adenocarcinoma cell lines was used to synthesize cDNA primed with oligonucleotides complementary to K-*ras* and Glucose-5-Phosphate Dehydrogenase (G6PD), which was used as an internal control. In almost all tumors expression of K-*ras* could be detected at levels about 5-fold higher than those of G6PD. From 7 adenocarcinomas with a mutation in K-*ras*, expression of both the mutated and wild-type alleles could be detected. Due to varying proportions of normal cells in the preparations, the level of wild-type expression could not be assessed adequately. However, expression of the mutated allele in tumors harboring the mutation was at least equal to the wild-type expression in all 7 cases.

To retrospectively study the clinical significance of K-*ras* point mutations in adenocarcinoma of the lung, we have applied to PCR to single 5 $\mu$m slices from standard formalin-fixed, paraffin-embedded tissue. We have now screened a total of 102 adenocarcinomas of the lung for this oncogene activation. All tumor samples were obtained after surgery with curative intent. No differences between the K-*ras* point mutation positive and negative groups were found with respect to age at diagnosis, stage and differentiation of the tumor, TNM classification, or the presence of previous or simultaneous tumors of other origin. Preliminary

survival data suggest a difference in prognosis as defined by the presence or absence of the point mutation in K-*ras*.

The pattern of point mutations within codon 12 differs between adenocarcinomas of different origin. The predominant point mutation in our study of lung tumors is a G to T transversion, either in the first or second position of codon 12 of K-*ras*. Taken together, this type of mutation accounts for over 80% of all detected mutations. It appears reasonable to assume that the differences between the type of mutations in human adenocarcinomas of different origin may reflect different patterns of exposure to tumor inducing agents. In this respect it is striking that of all investigated adenocarcinomas 12 came from non-smokers (12%), and that these were all negative for the mutation in K-*ras*. The association between smoking and the point mutations in K-*ras* is statistically significant (p-0.031). This finding suggests that the point mutations in K-*ras* were a direct result of exposure to carcinogenic ingredients in tobacco smoke. This link is, of course, supported by the reproducible induction of activating point mutations in *ras* oncogenes found in chemically induced animal tumors. Currently, we are investigating differences in resistance to irradiation and several cytostatic drugs between cell lines with and without the mutation in K-*ras*.

**CYTOKERATIN EXPRESSION OF MOUSE EPIDERMAL KERATINOCYTES DURING THE PROCESS OF NEOPLASTIC CONVERSION**

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Cytokeratins, the major cytoskeletal component of all epithelial cells, are differentially regulated. In general, they are expressed as pairs (type I-acidic and type II-basic) to form intermediate (10 nm) filaments. The role of this cytoskeletal component in the cells is a matter of speculation. It has been suggested its implication in signal transduction.

We are investigating the importance of intermediate filaments in mouse skin carcinogenesis, an animal model system based on the progressive induction of tumors (benign papillomas and malignant carcinomas) by treatment with initiating agents, as dimethylbenzanthracene, and promoters, generally TPA. In this system, the activation of a Ha-*ras* gene occurs during the initiation of carcinogenesis (Nature, 1986, 322:78-80).

Preliminary experiments indicated that the transfection into immortalized epidermal cells of a Ha-*ras* oncogene induced, in the transformed cells, the synthesis of new cytokeratins: K8 and K18, the natural pair of keratins in simple epithelium and in early stages of mouse embryogenesis.

The keratin analysis of several epidermal cell lines transformed by different routes or derived from skin tumors, all of which present different alterations in the Ha-*ras* gene, shows that expression of K8 and K18 is characteristic of cell lines which are tumorigenic in nude mice. Another interesting point is that, whereas the induction of K8 and K18 mRNAs is coordinately regulated in the different cell lines, a disbalance exists between K8 and K18 at the protein level; K18 is barely or not detectable at all by immunoblotting and immunofluorescence. However, K8 is forming filaments in the cells.

**INFLUENCE OF EXTRACELLULAR MATRIX AND CELL ADHESION MECHANISMS IN TUMOR PROGRESSION**

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Interactions of cells with one another and with extracellular materials are of vital importance for cell function, and they have major effects on proliferation, differentiation and organization of cells and tissues.

The concept of extracellular matrix has changed in the last years to a dynamic vision of the cell-substratum interactions; extracellular matrix components such as fibronectin interact with intracellular structures, such as the cytoskeleton, through specific cellular receptors and other "bridge"-proteins such as talin and vinculin. Molecules involved in intercellular interactions also interact with defined intracellular structures.

Several lines of evidence indicate that alterations of cellular interactions occur during the process of tumor progression, and new types of fibronectin are synthesized by transformed cells. The model of multistage tumor skin carcinogenesis offers an appropriate system to approach some aspects of cellular interactions in tumorigenic processes. This model is based on the progressive induction of tumors in mouse skin by treatment with initiating (chemical carcinogens, such as dimethylbenzanthrazene, DMBA) and promoting agents (such as phorbol esters, TPA). The tumors that arise are primarily benign papillomas, but some of these undergo further changes and progress to invasive carcinomas. In this system, activation of Ha-*ras* oncogenes occurs during initiation.

We are studying the influence of extracellular matrix components and calcium-dependent cell adhesion molecules in mouse skin carcinogenesis. We have observed that synthesis and expression of fibronectin is remarkably altered in carcinoma-derived cell lines, but only in some of them is it able to be organized in a defined extracellular matrix as detected by immunofluorescence studies. The specific type of fibronectin synthesized by the various cell lines is currently being investigated at the mRNA level. Expression of specific cell surface receptors is also being studied.

Studies on a Ca-dependent cell adhesion molecule, E-cadherin, indicates that the levels of expression of this protein are inversely related to the

tumorigenic potential of the various cell lines analyzed. This fact can be related to the recently proposed anti-invasin character of this protein.

The relationship of these components, fibronectin receptor and E-cadherin, with the cytoskeleton is presently being studied in collaboration with Dr. M. Quintanilla.

## RAS ONCOGENES IN CELL TRANSFORMATION AND HUMAN CANCER

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*Ras* oncogenes are members of a multigene family coding for proteins which are thought to act as signal transducers. Because activated *ras* oncogenes are frequently detected in primary tumors and apparently play a role in human cancer, *ras* oncogenes are among the best studied human oncogenes. The emerging evidence suggests that *ras* oncogenes can be involved in one or more steps of the multi-step process of cancer development. Thus, *ras* oncogene activation has been implicated in the distinct steps of cell immortalization, tumorigenic conversion, invasion and metastasis. However, the relationship between *ras* activation or overexpression and any of the above parameters in the multi-step process of carcinogenesis is likely to be influenced by other cellular factors. Evidence suggests a bifunctional role of *ras* genes either to block differentiation and stimulate cell proliferation or to induce differentiation and inhibit cell proliferation.

**MULTIPLE GENETIC ALTERATIONS IN SMALL-CELL LUNG CARCINOMA**

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It is now widely accepted that human carcinogenesis is a multi-step process involving more than one genetic alteration. Recent molecular genetic studies have shown that loss of genes at specific chromosomal loci occurs frequently in certain types of tumors, suggesting that recessive genetic changes are involved in the development of a variety of human cancers. Molecular analysis of genetic alterations with reference to the clinical and biological behavior of cancer cells will give us valuable information for understanding the molecular mechanisms involved in human carcinogenesis. We have performed an RFLP analysis for several different types of human cancers and detected loss of heterozygosity (LOH) at specific chromosomal loci in certain types of cancers. The results of our RFLP analysis will be summarized and presented with a specific emphasis on those of small-cell lung carcinoma (SCLC). LOH at 3 different chromosomal loci, 3p, 13q and 17p, was observed simultaneously in nearly 100% of SCLC. It was observed even in stage I tumors and an untreated tumor, and it occurs prior to NMYC amplification. The common region of LOH on 3p was 3p14-3p24.1, and this region was also frequently lost in carcinoma of the uterine cervix (100% at D3S2 on 3p14-21) as well as renal cell carcinoma (56% at ERBA on 3p22-24.1), suggesting the presence of tumor suppressor gene(s) for these cancers in this region. On chromosome 13, LOH was observed commonly in the region between 13q12 and 13q22, including the RB locus on 13q14, and normal RB protein was not detected in any of 9 SCLC cell lines by immunoprecipitation analysis. The common region of LOH on chromosome 17 was 17p13 and is the same as that in colon carcinoma and osteogenic sarcoma. An abnormal size of p53 transcript accompanied by the absence of normal p53 protein was observed in one of 9 SCLC cell lines. These results strongly suggest that critical target genes for the genesis of SCLC are the RB gene on 13p14, the p53 gene on 17p13, and an unidentified tumor suppressor gene on 3p14-24.21, and that at least six genetic alterations are necessary to convert a normal cell into a fully malignant cancer cell in the case of SCLC.

**HUMAN BREAST CANCER: IDENTIFICATION OF POPULATIONS WITH HIGH RISK OF RELAPSE IN RELATION TO ESTROGEN STATUS AND HER-2 OVEREXPRESSION**

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The knowledge of estrogen receptor levels in breast cancer constitutes a classical prognostic factor. Receptor-negative patients are at increased risk for relapse and shorter overall survival. However, among ER positive patients regarded as having a more favorable prognosis, we have recently defined a group present a high risk of relapse. This group of patients called ER+[R2], has been characterized by a ratio [ER-protein expressed in fm/mg of total protein] *over* [ER-mRNA expressed in pg/4 μg of total RNA] higher than 1.5. According to these results ER+[R] status has been assigned as an early prognostic factor (May *et al.*, 1989, Oncogene 4, 1037-1042).

On the other hand a frequently found alteration associated with breast cancer is an amplification of the HER-2 gene. A number of studies showed an association between amplification or overexpression of the HER-2 gene and poor prognosis in human breast cancer.

In an attempt to correlate the prognostic significance of ER+[R] status with HER-2 prognostic factor we have determined by Northern blotting the level of HER-2-specific mRNA in the series of 89 untreated breast cancer previously analyzed for the expression of ER-specific mRNA. HER-2 mRNA amounts displayed a wide range of values from 5 to 190 pg/4 μg of total RNA. High level of HER-2 mRNA (>20 pg/4 μg of total RNA) was correlated with i) inflammatory carcinoma, ii) presence of involved lymph nodes and iii) ER negativity. No significant correlation was seen between HER-2 mRNA level and age of the patient at diagnosis, tumor histological grade or ER+[R] status.

A multivariate analysis was performed to determine the interrelationship of HER-2 mRNA and ER+[R] status with disease-free survival. Results obtained with a median follow up of 24 months showed that ER+[R] status and HER-2 mRNA are two significant independent predictors of disease-free. With this relative short follow up lymph node involvement lost its significance on multivariate analysis.

Moreover, our study shows that HER-2 mRNA and ER-/ER+[R] status are additive as prognostic factors.

**EXPRESSION OF HPV16 TRANSFORMING PROTEIN E7 IN FISSION YEAST SCHIZOSACCHAROMYCES POMBE**

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The complete E7 open reading frame (ORF) of human papillomavirus type 16 (HPV16) was expressed in fission yeast *Schizosaccharomyces pombe* (*S. pombe*) under the control of a cloned yeast promoter. The HPV16 E7 protein synthesized in *S. pombe* is a phosphoprotein of the expected molecular weight (Mr - 17 Kd), which is recognized by antibodies raised in rabbits against MS2 DNA Polymerase E7 (MS2/E7) fusion protein. Immunofluorescence staining showed that HPV16 E7 phosphoprotein is localized in the nuclei of transformed *S. pombe*.

These results a) indicated that recombinant E7 protein synthesized by *S. pombe* is apparently undistinguishable from HPV16 E7 protein synthesized in higher eukaryotic cells expressing HPV16 genes; b) suggest that the phosphorylated, nuclear HPV16 E7 protein is synthesized in *S. pombe* in a form compatible with biological activity. The implications of these data for the study of HPV16 E7 gene function will be discussed.

**MHC ANTIGEN EXPRESSION AND ONCOGENE ACTIVATION ON TUMORS**

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Major histocompatibility complex molecules play an important role in self and non-self discrimination, antigen processing and peptide presentation to the immune system.

Any qualitative alteration in the expression of these polymorphic molecules will influence anti-tumor immune effector mechanisms like cytotoxic T cell responses or NK cell sensitivity.

It is becoming evident that mouse and human tumor cells have clones with different MHC class I and II expression. We have obtained in a chemical-induced fibrosarcoma (GR9 tumor) clones with different expression of class I H-2 molecules. These clones have a different behavior when injected "in vivo." Clones expressing high amounts of H-2 class I molecules are highly metastatic, NK-resistant and with low tumorigenicity. In contrast, those clones H-2-negative are low metastatic, NK-sensitive and highly tumorigenic. We have seen that H-2 class I transcription inversely correlated with c-*myc* RNA levels.

We have also demonstrated that 15% to 20% of human tumors completely lack HLA expression. In colon tumors, K-*ras* activation is absent in 100% of HLA-negative cases and the codon 12 point mutation is only present in HLA-positive tumors. In cell lines obtained from larynx tumors, we have observed similar results regarding c-*myc* and HLA class I transcription. Namely, c-*myc* RNA levels inversely correlated with HLA class I expression.

Finally, we have been able to correlate expression of HLA class I and HLA-DR with tumor prognosis emphasizing the role that MHC may play in anti-tumor immune response mechanisms.

# GENE AMPLIFICATION, TRANSLOCATIONS

D. SLAMON
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**ALTERATIONS OF THE erb2/neu PROTO-ONCOGENES IN HUMAN BREAST AND OVARIAN CANCERS**

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The HER-2/*neu* proto-oncogene encodes a putative growth factor receptor which was shown to be amplified in 25-30% of primary human breast cancers. Amplification of the gene was associated with disease behavior and was more predictive of outcome than any other known prognostic factor with the exception of positive lymph nodes in patients with Stage II disease. Since this initial report, there have been a number of studies evaluating both amplification rate and association between HER-2/*neu* and prognosis in breast cancer with considerable variation in published results. Some of this variability may be due to the small numbers of specimens studied or to significant differences in the methods used to evaluate the gene and its product. The current study addresses both biologic and clinical aspects of the gene and its alteration in human breast cancer. Sequence analysis of several c-DNA clones from actual tumors indicate that unlike the rat *neu* gene, mutations in the transmembrane domain may not be an absolute requirement for alteration of the gene. Instead, the data are consistent with an alteration involving overexpression of a normal product since gene amplification is virtually always associated with high levels of expression in the malignant cells of these tumors. The gene product has a half life of 12 hrs and can be readily identified in both tissue homogenates and tissue sections from breast tumors. To circumvent potential statistical problems introduced by small numbers reported in earlier studies, an analysis of HER-2/*neu* amplification was done in 668 primary tumors, 526 of which had long term follow-up. The overall amplification rate in breast cancer as well as the association between gene amplification and disease outcome previously reported for Stage II patients was confirmed in this larger series. A comprehensive analysis of the gene ad its products (DNA, RNA and protein) was simultaneously performed on 187 tumors for which sufficient tissue was available. This analysis identified several potential shortcomings of the various method used to evaluate HER-2/*neu* in this disease (i.e., Southern, Northern, and Western blots, and immunohistochemistry). The Western blot was most prone to errors in assessing HER-2/*neu*, while immunohistochemical analysis of frozen tissue was the most reliable method. This result has diagnostic implications. The data presented in this study further supports the concept that the HER-2/*neu* gene may play a role in the pathogenesis of some human breast cancers.

## MULTIDRUG RESISTANCE IN TUMOR CELLS: MOLECULAR GENETICS AND DIAGNOSTICS

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The ability of mammalian cells to survive exposure to different lipophilic cytotoxic drugs, including some of the most common anti-cancer agents, is known as multidrug resistance. This type of resistance results from increased expression of genes designated *mdr*. Increased *mdr* expression in some cases is a consequence of gene amplification. The human *mdr* gene family includes two members, MDR1 and MDR2, which are linked within 300 kb of DNA on chromosome 7q21.1. Although both genes encode very similar proteins, only MDR1 expression has been associated with multidrug resistance. The MDR1 gene codes for a 170 Kd membrane protein (P-glycoprotein), which appears to function as an ATP-dependent efflux pump, providing for decreased drug accumulation in multidrug-resistant cells. P-glycoprotein consists of two similar halves, each half including six predicted transmembrane segments and a nucleotide-binding site. Site-directed mutagenesis studies indicate that both nucleotide-binding sites are needed for normal drug efflux by P-glycoprotein. P-glycoproteins are related to a large group of nucleotide-binding proteins associated with active membrane transport in pro- and eukaryotic cells. Some multidrug-resistant cell lines, while expressing the same MDR1 gene, show altered patterns of cross-resistance to different drugs. In one such cell line, preferential resistance to its selective agent, colchicine, was found to result from a point mutation in the MDR1 gene, leading to a single amino acid substitution. This change resulted in altered drug binding and transport by the mutant P-glycoprotein.

In normal human tissues, the highest levels of P-glycoprotein expression are observed in the adrenal cortex, at lumenal surfaces of the kidney, liver and gastrointestinal tract, as well as in placental trophoblasts and endothelial cells in the brain and testes. In most normal tissues, the expressed P-glycoprotein appears to be encoded by the MDR1 gene. The MDR2 gene is expressed at a high level in the liver and at low levels in kidney, spleen and the adrenal gland. MDR1 expression has been detected in many different types of human tumors before and after chemotherapy, suggesting an association with both the intrinsic and the acquired forms of clinical drug resistance. The MDR2 gene, which is not associated with multidrug resistance, is also frequently expressed in different

tumors, including those that do not express MDR1. Detection of MDR1 mRNA or P-glycoprotein provides a potential diagnostic tool for multidrug resistance in clinical cancer. Any useful assay, however, should be capable of detecting low levels of MDR1 expression, which are sufficient for significant levels of resistance, and it should clearly discriminate between the products of MDR1 and MDR2 genes. We have found that enzymatic amplification of mRNA sequences by polymerase chain reaction provides a particularly sensitive, specific and quantitative diagnostic procedure. In collaboration with R. S. Weinstein, we have noticed by using *in situ* assays that MDR1 expression in untreated solid tumors often appears to be heterogeneous, with only a minority of cells expressing MDR1 mRNA or P-glycoprotein. In colon tumors, P-glycoprotein-expressing cells are preferentially localized in the areas of apparent invasive growth. The minimal levels of MDR1 expression or frequencies of MDR1-positive cells associated with clinically refractory tumors remain to be determined. Elucidation of the mechanism of multidrug resistance suggests several clinical strategies for circumventing this type of resistance to cancer chemotherapy.

**BCR/ABL IN Ph-POSITIVE LEUKEMIA**

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The Philadelphia (Ph) chromosome is an acquired abnormality found in the leukemic cells of patients with either acute lymphoblastic leukemia (ALL) or chronic myelogenous leukemia (CML). This chromosome is the result of a reciprocal translocation between chromosomes 9 and 22. In CML, translocation breakpoints occur within the ABL proto-oncogene on chromosome 9 and the BCR gene on chromosome 22. The translocation results in the production of a chimeric BCR/ABL mRNA that is translated into a fusion protein. This protein, P210, exhibits a tyrosine-protein kinase activity differing from that of the normal ABL gene product.

More than 95% of CML patients have either a standard or variant Ph-translocation and, therefore, this abnormality is used cytogenetically as a diagnostic marker. Since more than 95% of CML patients have a breakpoint within a region encompassing only 5.8 kb of DNA (in the major breakpoint cluster region, Mbcr) of the BCR gene, DNA probes in combination with Southern blot analysis can be used to detect the Ph-chromosome.

The Ph-chromosome is not nearly as common in ALL: only 17%-25% of adults and 2%-6% of children have been reported to have this translocation. Cytogenetically, all Ph-chromosomes appear similar, however at the molecular level, Ph-positive ALL patients appears to constitute a heterogeneous group; some patients have an Mbcr breakpoint as found in patients with CML, whereas others lack a breakpoint in this region. In this latter group, the ABL oncogene was found to be involved in the translocation since an abnormal ABL protein (P190) was detected. The BCR gene was also implicated since the P190 consists of amino terminal BCR exon 1-encoded residues fused with a carboxy terminal ABL moiety. Recently, the genomic sequences of the entire BCR gene have been cloned and therefore it is now feasible to systematically examine the entire gene for rearrangements in Ph-positive leukemia. Initial data suggest that the molecular genetic analysis results in a distinction between different age-groups of Ph-positive ALL and the presence of the P210 ad P190.

Although the presence of the Ph chromosome is used as a diagnostic marker and the BCR/ABL fusion protein is invariably found in Ph-positive patients, very little is known about the direct role, if any, of BCR/ABL in the pathogenesis of leukemia. Since *in vivo*, the BCR/ABL translocation most probably occurs in a hematopoietic stem cell, it would be desirable to create an *in vivo* model to study the effects of the activated BCR/ABL protein in affected hematopoietic cells. One method to introduce the chimeric gene into all mouse tissues is through the generation of transgenic mice. Transgenic mice have proven to be quite valuable in evaluating the *in vivo* tumorigenic potential of activated cellular oncogenes.

We have introduced the P210 encoding BCR/ABL gene, under control of the BCR promoter, into transgenic mice; our results point to a pleiotropic lethal effect on embryogenesis. In addition, experiments were performed with the P190 producing BCR/ABL hybrid gene driven by the metallothionein promoter; our results dramatically demonstrate that hematopoietic expression of the BCR/ABL gene is causally associated with the rapid induction of acute leukemia in very young (10-58 days) transgenic mice.

## SUMMARY

**M. PERUCHO**
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**SUMMARY**

The workshop was organized in four different sessions, centered in the fields of *ras* oncogenes, tumor suppressor genes, and nuclear oncogenes, with other topics covered by individual talks including oncogene amplification, chromosomal translocations, DNA tumor viruses, and multidrug resistance. Due to size and time constraints, studies on cancer diagnosis by immunological detection of tumor markers (monoclonal antibodies) were deliberately excluded. At the end of the workshop, Mariano Barbacid gave an excellent summary of the highlights of the workshop. What follows is an attempt to recapitulate the main results presented by the speakers in the workshop.

In the session on *ras* oncogenes, F. McCormick gave a talk on the biochemistry of *ras* proteins and their association with the GTPase activating protein (GAP). The new discoveries of Frank's group appear to link two classes of oncogene products via GAP. Thus, *src* phosphorylates GAP in tyrosine, which is then inhibited in its stimulating action on the GTPase activity of *ras*, the final result being *ras* activation. The linkage of the tyrosine kinase membrane receptors with the signal transducing *ras* proteins through GAP might lock into place the oldest of the oncogene products, p60 *src*, which has resisted the unraveling of its oncogenic mechanism of action for more than 20 years. However, the puzzle is still incomplete, since it is not clear which is the link in the chain downstream of *ras*. If it is the same GAP, or another protein, it remains to be connected with the nuclear oncogenes or tumor suppressor genes to fill, at least conceptually, the pathway from extracellular signal to cell replication (see Introduction). In addition, F. McCormick also reported recent experiments indicating that G proteins also possess oncogenic potential, activated by somatic point mutations in some pituitary tumors exhibiting elevated levels of cAMP.

Angel Pellicer described his studies in both animal and human tumors linking H-*ras* activation with benign, self-regressing keratoacanthomas and malignant squamous cell carcinomas. The mutational data suggest a differential role of the *ras* gene in these types of tumors, both in rabbits and in humans. In addition, Angel gave an updated report on his studies on radiation (x-rays and neutrons)-induced thymomas in mice, and his efforts to determine the extent

of involvement in carcinogenesis of the new mutation at codon 146 of the c-K-*ras* gene previously identified in a neutron-induced tumor.

Johannes Bos summarized his studies on the involvement of *ras* activation in a wide spectrum of human tumors. His analysis adds a new dimension to the issues of tumor heterogeneity as well as the origin of the precursor cell of some leukemias, as they can now be approached because of specific differences at the genetic level, albeit so small a change as a single point mutation. Hans' extensive studies on *ras* involvement in human cancer provide a diagnostic tool which undoubtedly will eventually be of importance for cancer therapy.

M. Perucho reported his studies on the expression and mutational activation of the c-K-*ras* gene in human colo-rectal and pancreatic carcinomas. The data point to the enormous complexity of the system, as given by the multiple types of mutations and the accumulative alterations in *ras* allelic dosage, apparently playing a role in tumor progression. Provocative associations were reported between presence or absence of *ras* mutations and/or their nature and a number of clinical parameters such as the age of the cancer patients and the histological type of the tumors.

As reported in the four talks of the *ras* oncogene session, the analysis of *ras* activation has reached a high sophistication in the technical aspects (mainly due to the use of *in vitro* gene amplification by the polymerase chain reaction (PCR)), which offer new and complementary approaches for cancer diagnosis, including early cancer detection.

In the session of tumor suppressor genes, Bob Weinberg gave one of his habitually enlightening and entertaining talks on the molecular genetics of the retinoblastoma gene (Rb), the first tumor suppressor gene described and isolated. The important role that the gene plays in cancer development was stressed by the widespread distribution of tumors (osteosarcomas, small cell carcinomas of the lung, and bladder carcinomas, among others) exhibiting alterations in the Rb locus, besides the initial childhood retinoblastoma.

The importance of the Rb product was further documented by Ed Harlow's studies on the biochemistry of its interaction with the transforming proteins

of DNA viruses such as SV40, polyoma, adenovirus, and papillomavirus. It appears now that DNA tumor viruses induce the transformed phenotype by functional inactivation of the products of these growth suppressor genes, the Rb gene and the recently characterized p53 gene, and possibly other proteins as suggested by Ed's data. Thus, the insight of attacking the problem of malignant transformation of the eukaryotic cell by studying the DNA tumor viruses is finally going to pay off.

Eric Fearon reported the tour de force of Bert Vogelstein's group in the analysis of the genetic alterations underlying the development and progression of colo-rectal cancer. Besides their identification of *ras* mutations and their original description of allelic deletions and mutational inactivation of the p53 gene, which appear to be even more widespread than the Rb gene, in colon as well as lung, brain, breast and other tumors examined to date, Eric gave an impressive description of the identification and isolation of another putative tumor suppressor gene, located in chromosome 18. If the preliminary observation turns out to be correct, that is, that the gene encodes a phosphatase, then the process will become a full circle: oncogenes as tyrosine protein kinases, and anti-oncogenes as phosphatases. In that case, these studies together with the predictable generalization of Frank McCormick's findings, will represent milestones in modern cancer research.

The nuclear oncogenes session provided basic lectures by Fred Alt and Prem Reddy on the molecular biology of c-*myc* and c-*myb*, respectively. The use of transgenic mice and gene targetting by homologous recombination to study oncogene function was well documented in Alt's talk. Preliminary results presented by Prem Reddy might fill the long awaited gap on the functional role of c-*myc* as he described evidence showing its specific DNA-binding ability. Talks by Manfred Schwab and Riccardo DallaFavera delved more into the diagnostic and prognostic value of genetic alterations of the *myc* gene family for neuroblastomas and a variety of leukemias, respectively. Manfred recapitulated the first evidence for the prognostic value of N-*myc* amplification for neuroblastoma and Riccardo described a strikingly high incidence of clustered mutations at the first exon-intron region of c-*myc* in B-cell malignancies. In addition, mutational activation of *ras* (mainly N-*ras*) and activation of c-*myc* and bcl-2 by chromosomal translocations in non-Hodgkin lymphoma (NHL), acute lymphoblastic leukemia (ALL),

AIDS-associated NHL and in follicular lymphoma and diffuse lymphoma were also reported. Together with J. Bos', these studies are pioneering the molecular classification of human leukemias, and eventually they should influence the choice of therapeutic treatment for this heterogeneous spectrum of neoplastic diseases.

The topic of chromosomal translocations involved in leukemogenesis was further developed by John Groffen for the BCR-ABL chimeric gene result of the reciprocal translocation between chromosomes 9 and 22. This is the underlying molecular genetic lesion responsible for the Philadelphia chromosome (the first cytogenetic abnormality associated with a human malignancy), which occurs in practically all chronic myelogenous leukemias (CML) and in some of the acute lymphoblastic leukemia (ALL). Development of molecular probes to detect this translocation is one of the first examples of the application of molecular genetics for cancer diagnosis, albeit so far restricted to specialized reference laboratories. John's results with transgenic mice points to a single hit neoplasia induced by the fused BCR/ABL gene. Whether this is the case for the human disease remains to be seen.

Gene amplification was the topic of two of the last talks of the workshop. Dennis Slamon reported his data on the involvement and prognostic value of amplification and overexpression of the *Erb*-2/*neu* protooncogene in human breast and ovarian cancers. Screening of over 500 tumors with their corresponding follow-up confirmed Dennis' pioneering studies in both rate of oncogene amplification as well as disease outcome. Together with N-*myc* amplification in neuroblastoma, the amplification of the *Erb*-2/*neu* oncogene in breast and also in ovarian cancers are the first well characterized examples where diagnostic approaches at the molecular level offer an additional value for cancer prognosis.

Igor Roninson gave an extensive overview on the fascinating phenomenon of multidrug resistance (mdr), which has important implications for cancer chemotherapy. Increased expression of mdr genes, which is sometimes a result of gene amplification, is the mechanism by which cells become resistant to exposure to different lipophilic cytotoxic drugs. We learned the mechanisms of the basic process as well as those involved in the appearance of increased resistance after exposure to specific drugs, which involve somatic point

mutations in what appears to be a hypermutable gene region. Detection of mdr mRNA or its encoded P-glycoprotein may provide diagnostic tools for multidrug resistance in clinical cancer, and Igor described his results using the polymerase chain reaction for amplifying mRNA sequences and his efforts to quantitate these experiments.

Another isolated topic was the involvement of human papilloma viruses in various types of human cancer, originally described by Harald zur Hausen for cervical carcinoma. Ethel-Michelle de Villiers reported the existence of over 50 different viral species, some of which are important etiological agents for anogenital as well as oral and respiratory tract cancers. Efforts to understand the basis of this cancer etiology are being enormously facilitated by the findings described by Ed Harlow on the interaction of E6 and E7 transforming proteins of papilloma virus with the retinoblastoma and the p53 tumor suppressor gene products. The detection of papilloma sequences in biopsies by *in situ* hybridization or by enzymatic amplification by the PCR are providing new approaches for diagnosis of premalignant and high risk stages.

The session of short communications recapitulated the entire workshop because of the topics covered. Talks by J. C. Lacal and M. Quintanilla dealt with some basic aspects of p21 *ras* biochemistry and its effects on other cellular proteins in animal tumor model systems. R. Slebos reported the up-to-date data on the incidence of *ras* mutations in human lung cancer with the interesting association between cigarette smoke and c-K-*ras* mutations and the presence of mutated *ras* genes with the worst prognosis of lung adenocarcinoma. D. Spandidos summarized the studies on the *in vitro* transformation by mutated *ras* and on clinical samples by immunohistochemistry.

Special mention is deserved by the communication by Jun Yokota from the National Cancer Center in Tokyo, on the search for genetic alterations in small cell carcinoma of the lung. In a convincing series of experiments, Jun reported the presence of allelic deletions in practically the totality of samples examined for three loci at chromosomes 3, 13, and 17, the last two involving the Rb and p53 anti-oncogenes, the first not yet characterized. In addition to *myc* gene family amplification, the deletions observed by the Japanese group imply that

at least six genetic alterations are required for development of small cell carcinoma of the lung.

Talks on the association of the *erb2/neu* gene amplification with estrogen receptor (HER) by E. May, expression of yeast of papilloma virus transforming protein by M. Tommasino, and on MHC antigen expression and oncogene activation by F. Garrido completed the topics covered in the workshop.

In conclusion, the workshop conveyed successfully its central theme on the recent advances in cancer diagnosis through animated discussions between the speakers and participants. In addition, it provided an overview on the most recent developments on oncogene and tumor suppressor gene research. Particularly fortunate in terms of timing, there were two reports of potential importance, that of the linkage between protein kinases and *ras* via GAP and of the isolation of the novel putative tumor suppressor gene in chromosome 18 deleted in the majority of colo-rectal carcinomas. The purpose of the workshop was therefore fully accomplished and it is expected that the diffusion of these findings between the Spanish participants will be very positive for their ongoing and future research efforts in this exciting field of cancer molecular genetics.

## LIST OF PARTICIPANTS

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240 **Genetic Strategies in Development.**
Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon y F. Jacob.

244 **Course on Genome Evolution.**
Organized by E. Viñuelas. Lectures by R. F. Doolittle, A. M. Weiner/N. Maizels, G. A. Dover, J. A. Lake, J. E. Walker, J. J. Beintema, A. J. Gibbs, W. M. Fitch, P. Palese, G. Bernardi y J. M. Lowenstein.

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

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

248 Miguel Beato
**Course on DNA - Protein interaction.**

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