HUMAN TUMOR VIRUSES

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Cellular Targets of DNA Tumor Viruses

NZ 001 A ROLE FOR THE E2F PRODUCT IN REGULATING CELL CYCLE PROGRESSION., Joseph R. Nevins, Howard Hughes Medical Institute, Section of Genetics, Duke University Medical Center, Durham, North Carolina 27710.

The recent isolation of the E2F1 cDNA clone has now allowed a more detailed analysis of the role of the E2F transcription factor in cellular transcription control and the regulation of E2F function by Rb and the Rb-related p107 protein. Given the potential role of E2F in activating cellular genes such as DHFR and thymidine kinase during the G1/S phase transition, we have assayed for the ability of E2F1 to alter cellular growth control. We find that over-expression of the E2F1 cDNA can activate DNA synthesis in cells that would otherwise growth arrest, with an efficiency that is similar to that achieved by the expression of the adenovirus E1A gene. Moreover, microinjection of the E2F1 cDNA into quiescent cells can induce S phase entry as measured by BrdU incorporation. Two E2F1 mutants, which are unable to trans-activate the DHFR and TK promoters, are also unable to induce S phase. Moreover, prevention of E2F synthesis following serum stimulation of quiescent cells through the use of an E2F1 antisense olgionucleotide blocks S phase entry. We conlcude that the E2F transcription factor plays an important role in progression into S phase and that this likely coincides with its capacity to stimulate transcription.

NZ 002 TRANSCRIPTIONAL ACTIVATION BY THE ADENOVIRUS E1A PROTEIN: ANTI-REPRESSION, Anny Usheva[1], Nobuo Horikoshi[1], Brian Lewis[1], Roberto Weinmann[2], and Thomas Shenk[1], [1]Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014 and [2]Bristol-Meyers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543-4000.

The adenovirus E1A proteins activate transcription by binding to cellular proteins and altering their function. Two examples will be discussed in which E1A proteins bind to cellular factors and relieve transcriptional repression. The first involves the TATA-binding protein. The tumor suppressor, p53, activates transcription when it binds to a promoter at a p53 response element, and, in promoters that lack a response element, p53 can bind to the TATA-binding protein and repress transcription. 13S E1A protein, like p53, also binds to the TATA binding protein; but, in contrast to p53, it activates transcription. The 13S E1A protein displaces p53 from the TATA-binding protein, and it can relieve p53-mediated repression both within transfected cells and within cell-free transcription extracts. Thus, part of the mechanism by which the E1A protein activates transcription through the TATA-binding protein involves its ability to block the activity of a cellular repressor. The second example involves a cellular transcription factor termed YY1. YY1 binding sites can function as initiator elements at which YY1 directs RNA polymerase II to the promoter. YY1, TFIIB and RNA polymerase II are sufficient to reconstitute transcription on a supercoiled template DNA containing a YY1 initiator element. When YY1 binding sites are located upstream of the transcriptional start site, YY1 can repress transcription. Both 12S and 13S E1A proteins bind directly to YY1 and block its ability to repress transcription.

Cellular Proliferation

NZ 003 HEPATITIS B VIRUSES AND LIVER CANCER: THE WOODCHUCK MODEL, Marie Annick Buendia, Geneviève Fourel, Yu Wei, Catherine Transy, and Claire Angelique Renard, Unité de Recombinaison et Expression Génétique, INSERM U163, Institut Pasteur, Paris, France.

Hepatitis B virus (HBV) has been associated with the development of hepatocellular carcinoma (HCC) by extensive epidemiological studies, but the underlying mechanism of liver cell transformation has not been defined. Other arguments linking HBV and HCC come from naturally occuring models: the woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV), two closely related viruses of the hepadna group, induce chronic hepatitis and a high frequency of liver tumors in native hosts.

Our recent studies have pointed out the predominant role of *myc* family genes in the development of liver cancer in these rodent species. Amplifications of the c-*myc* locus were commonly observed in HCCs from GSHV-infected animals. Tumors induced by WHV are characterized by frequent rearrangements of c-*myc*, N-*myc*, and predominantly N-*myc*2, an intronless oncogene recently identified in the woodchuck and squirrel genomes. Insertional activation of *myc* genes by WHV DNA was demonstrated in about 50% of cases, and a mechanism of enhancer insertion was evoked, similar to that observed in retroviral insertions into *myc* genes in murine T lymphomas. The high oncogenic impact of the viral insertion events was recently demonstrated by the development of liver cancer in different lines of transgenic mice carrying a mutated c-*myc* or N-*myc*2 gene and adjacent WHV sequences isolated from woodchuck HCCs.

Most woodchuck hepatomas showing only wild type c-*myc* and N-*myc* genes harbored integrated viral sequences and produced abundant N-*myc*2 mRNAs. Within this group, 4 independent tumors carrying single integration events were selected and genomic libraries were constructed. Restriction mapping of the WHV-positive clones showed that the 4 viral inserts were clustered in the same cellular locus, less than 20 kb apart. In situ hybridization of chromosome preparations from woodchuck fibroblasts mapped the N-*myc*2 gene and the new insertion locus to the same region, at chromosome X q22-23. The new locus, termed WIN (for WHV Insertion site related to N-*myc*2) was localized more precisely at a 190-200 kb distance of N-*myc*2, on the 3' side of the gene by pulse-field electrophoresis. Integrations of viral DNA, including in all cases the WHV enhancer, were detected in WIN on Southern blots in more than one half of woodchuck tumors showing no rearrangement of any *myc* gene. Further analyses of a 40 kb region spanning the normal WIN allele failed to reveal active transcription of the locus in tumor and liver samples.

Noticeably, tumors arise earlier and more frequently in WHV-infected woodchucks than in other hepadnavirus systems. The marked oncogenicity of the woodchuck virus may be related to a unique ability of this virus to provoke insertional activation of *myc* genes, either directly or by a long-range effect of the viral enhancer. These data might lead to re-evaluate the importance of viral integration in HBV-related liver cancer in humans.

Viral and Cellular Control of Cell Cycle and Replication (Joint)

NZ 004 p16^{INK4}; AN SPECIFIC INHIBITOR OF THE CDK4/CYCLIN D KINASE, David Beach, Manuel Serrano and Gregory J. Hannon. Howard Hughes Medical Insitute, Cold Spring Harbor Laboratory, NY 11724.

Passage through the cell cycle in mammalian cells requires a group of related kinases known as cyclin-dependent kinases or CDKs whose activity and substrate specificity depends on their association with a family of positive regulatory subunits known as cyclins. The complexes formed by CDK4 and the D-type cyclins have been implicated in the control of cell proliferation during the G1 phase of the cell cycle. In normal, proliferating, cells, CDK4 associates with D-type cyclins and with a protein of 16 KD molecular weight, p16^{INK4} (inhibitor of CDK4). In human cells transformed with viral oncoproteins that inactivate the Rb tumor suppressor protein, p16^{INK4} is overexpressed being the main, if not exclusive, partner of CDK4. We have isolated a human cDNA clone encoding p16^{INK4} by using the two-hybrid screening system in yeast with CDK4 as the target protein. We have found that p16^{INK4} specifically associates with CDK4 *in vitro* and *in vivo*, and does not associate with other CDKs. We have used extracts from insect cells overexpressing CDK4 and D-type cyclins to reconstitute active CDK4 kinase; when these extracts are incubated with p16^{INK4} the kinase activity of CDK4 toward Rb is completely inhibited. Inhibition only occurs when using CDK4, and not CDK2, as catalytic subunit. In normal cells the balance between p16^{INK4} and D-type cyclins could determine the level of CDK4 kinase activity. Inactivation of Rb during the mid-G1 phase is thought to be fulfilled by CDK4 associated with D-type cyclins. In transformed cells expressing viral oncoproteins, such as T-antigen, E1A or E7, Rb is constitutively inactivated and the G1/S control is, at least, partially disrupted. We propose that the disruption of the G1/S control is sensed by a signalling pathway that upregulates the expression of p16^{INK4}, therefore inhibiting the CDK4 activity. In the particular case of cells expressing viral oncoproteins, this negative loop is unable to restore the Rb-dependent G1/S control because the Rb function in these cells is constitutively abrogated.

NZ 005 REGULATION OF THE STRUCTURE AND FUNCTION OF WILD-TYPE AND MUTANT FORMS OF p53. Jill Bargonetti, Xinbin Chen, George Farmer, Philip Friedlander, Lata Jayaraman, Jim Manfredi, Scott Miller, Yan Wang and Carol Prives. Department of Biological Sciences, Columbia University, N.Y., N.Y. 10027

The p53 tumor suppressor protein is a DNA binding transcriptional activator. Levels of wild-type p53 increase in cells after DNA damage and, depending on cell type or experimental conditions, this leads to either cell cycle arrest in G1 or apoptosis. It is likely that the ability of wild-type p53 to activate transcription is intrinsic to its ability to arrest cells. We have characterized the role of p53 as a transcriptional activator with respect to its interactions with the basal transcription factor TFIID (TBP). Our data indicate that TBP/TFIID and p53 cooperate to bind to DNA and activate transcription *in vivo* and *in vitro*. We are interested in the structure and function of wild type and mutant forms of p53. We have analyzed the domains of p53 that are involved in its DNA binding properties. Our data indicate that the central conserved region where the tumor derived mutations are located contains the sequence specific DNA binding domain. The C-terminal portion of the protein contains a region that binds non-specifically to DNA and is capable of reannealling complementary single strands of DNA. Additionally, the N-terminal and C-terminal regions of p53 contain sequences as well as phosphorylation sites that regulate the DNA binding properties of the protein. We have observed that the majority of tumor-derived p53 mutants are conditionally defective for DNA binding and display DNA binding at temperatures lower than 37° C. Therefore identifying means to stabilize the binding of mutant p53 proteins at 37° C is an important goal with therapeutic implications.

Lymphocyte Proliferation and EBV

NZ 006 IMMORTALIZING GENES OF EPSTEIN-BARR VIRUS, Bill Sugden, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706

Epstein-Barr Virus (EBV) infects human B-lymphocytes and induces and maintains them in a proliferating state such that infected cells eventually efficiently yield immortalized progeny. Genetic analyses of EBV that either mutate individual genes or remove large tracts of viral DNA have identified viral genes that contribute to the process of immortalization. Complementary studies have identified activities of viral gene products that also contribute to immortalization. Two such immortalizing genes of EBV are the BKRF-1 orf that encodes EBNA-1 (EB nuclear antigen-1) and BNLF-1 orf that encodes LMP (latent membrane protein). EBNA-1 functions to support EBV plasmid DNA replication and activate transcription. We have made dominant negative mutants of EBNA-1 and are using them to analyze the distinct functions of this viral gene. LMP behaves as an oncogene in established rodent cells and affects several phenotypes in different cell lines. We have built on recent observations to study induction of NFκB activity by the transient expression of LMP and its mutant derivatives. These studies indicate that LMP does induce NFκB activity soon after its introduction into some cell lines, that the induction of this activity may account for some of the phenotypes associated with the expression of LMP, but cannot account for them all.

Papillomaviruses and the Epithelial Cell

NZ 007 GROWTH FACTOR RECEPTORS AS CELLULAR MEDIATORS OF PAPILLOMAVIRUS TRANSFORMATION, Daniel DiMaio, Lisa Petti, Daniela Drummond-Barbosa, Laura Nilson, Eun-Seong Hwang, and Carl Henningson, Department of Genetics, Yale University School of Medicine, New Haven, CT 06510.

The E5 proteins of the papillomaviruses are short, hydrophobic proteins that can transform established cell lines to tumorigenicity. The 44-amino acid bovine papillomavirus E5 protein (BPV-E5) efficiently transforms rodent fibroblasts and serves as the prototype papillomavirus E5 protein for genetic and biochemical studies. In transformed fibroblasts, BPV-E5 expression activates cell surface and intracellular forms of the endogenous PDGF ß receptor as assessed by several biochemical measures including increased receptor tyrosine phosphorylation. Moreover, the E5 protein and the activated PDGF receptor form a stable complex in transformed cells, implying that PDGF receptor activation is mediated by binding of BPV-E5 and the receptor. Although BPV-E5 can also form complexes with other growth factor receptors when they are over-expressed in COS monkey cells, the PDGF receptor appears to be the preferred endogenous target of the BPV-E5 protein in transformed bovine and rodent fibroblasts. BPV-E5 has also been reported to interact with an α-adaptin-like molecule and the 16kDa subunit of the vacuolar H^+-ATPase, proteins that might influence receptor metabolism or activity. In gene transfer experiments with epithelial and lymphoid cells that normally lack the PDGF receptor, a wild type PDGF receptor but not a tyrosine kinase negative mutant can deliver a proliferative signal in response to BPV-E5 expression. These results provide compelling evidence that BPV-E5 causes sustained activation of the PDGF receptor tyrosine kinase which in turn initiates its growth stimulatory signal transduction cascade. In contrast, the human papillomavirus (HPV) E6 and E7 transforming proteins appear to transform cells by inhibiting cellular growth suppressor proteins. The expression of BPV-E5 in transformed basal keratinocytes in bovine fibropapillomas and the ability of BPV-E5 and HPV16-E5 to transform epidermal keratinocytes imply that papillomavirus E5 proteins affect growth control of keratinocytes as well as fibroblasts. We are testing whether the cellular targets of papillomavirus E5 proteins in keratinocytes are also growth factor receptors.

NZ 008 **GENETIC AND CELLULAR CONTROL OF KERATINOCYTE TRANSFORMATION,** Enzo Calautti[1], Janice Brissette[1], Nalin Kumar[2], Norton Gilula[2], Caterina Missero[1], & G. Paolo Dotto[1], [1] Cutaneous Biology Research Center, Harvard University, MGH East, Charlestown, Massachusetts, and [2] Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California.

Lining epithelia are characterized by a very high turnover rate and a fine balance must exist between growth of their cells and differentiation. Mouse primary keratinocytes provide an ideal system to study control of these processes, both under normal conditions and during transformation. Addition of calcium to these cells triggers a terminal differentiation program which closely resembles that of keratinocytes in the upper epidermal layers in vivo. Some of these changes - but not others - are also induced by treatment with the tumor promoter TPA.

We have previously shown that tyrosine phosphorylation of a specific set of proteins occurs as a very early event in calcium- and TPA-induced differentiation and is required for these processes to occur. In particular, a single protein of ~80 kD is tyrosine phosphorylated in response to both calcium and TPA, while another of ~60 kD is tyrosine phosphorylated only in response to calcium. This latter protein was recently identified as a p62 protein which associates with the ras-GTPase activating protein (ras-GAP).

Nothing is known about the tyrosine kinases involved in keratinocyte differentiation. We report here that a complex pattern of tyrosine kinase activation exists, which is distinct for calcium- and TPA-induced differentiation and involves new as well as well characterized enzymes.

In a second set of studies, we are investigating gap junctional intercellular communication (GJIC) in mouse primary keratinocytes under basal conditions, transformation and calcium- and TPA-induced differentiation. Our most recent work indicates that calcium-induced keratinocyte differentiation is associated with a specific switch in gap junction protein expression and this in turn controls the specificity of transfer of second cell messengers. The possibility that selective gap junctional communication plays an important role in keratinocyte growth/differentiation control will be discussed.

Hepatotropic Viruses: Transcription and Replication

NZ 009 REPLICATION AND ASSEMBLY OF HEPATITIS B VIRUSES P. Ostapchuk*, J. Lingappa, P. Hearing* and D. Ganem, HHMI and University of California, San Francisco, CA 94143 and State University of New York at Stony Brook, NY

The hepatitis B viruses are enveloped, icosahedral DNA viruses whose assembly proceeds via budding of preformed, cytoplasmic nucleocapsids into the endoplasmic reticulum. Nucleocapsid formation involves the self-assembly of 180 monomeric subunits of the viral C protein, together with incorporation of viral RNA and the polymerase (P). P protein is required for RNA packaging but not capsid assembly; packaging involves recognition of an RNA stem-loop either directly or indirectly by P. We have reproduced the capsid assembly reaction in vitro, by expressing C mRNA in cell-free translation systems. Assembly proceeds through several distinct intermediates culminating in particles indistinguishable from authentic viral capsids by equilibrium density sedimentation. Using this system we have investigated the role of a cytosolic chaperonin in multimer assembly. A TCP-1 related 60kD protein is found in association with C polypeptide in two different forms of assembling capsid subunits, but is not associated with either the initial unassembled C polypeptide or with the final product that comigrates with authentic assembled capsid. These data point to a role for cytosolic chaperonins in the assembly of multimeric viral capsids, and further suggest that beyond their presumed roles in assisting the folding of monomeric precursors they may also participate in subsequent interchain interactions.

We have also examined the acquisition of envelope proteins by cores during budding. At least two related envelope proteins must be acquired during this step. By examining the transmembrane structure of one of these proteins before and after virion assembly we show that a dramatic rearrangement in the protein topology of the viral envelope must occur during or immediately following budding. This may be due to protein or lipid reorganization, or both.

NZ 010 REPLICATION OF HEPATITIS DELTA VIRUS RNA: HOW DOES RNA POLYMERASE II DO IT?, Michael M. C. Lai and Mei Chao, Howard Hughes Medical Institute, Department of Microbiology, University of Southern California School of Medicine, Los Angeles, CA 90033.

Hepatitis delta virus (HDV) coinfects with hepatitis B virus (HBV) and causes fulminant hepatitis. It has a tendency to lead to chronic infection and may shorten the latency period for the development of hepatocellular carcinoma. HDV contains a single-stranded circular RNA genome of 1.7 kb, and encodes a hepatitis delta antigen (HDAg). Two species of HDAg have been detected in every patient's serum and liver: the small HDAg (195 amino acids) trans-activates HDV RNA replication, while the large HDAg (214 amino acids) causes dominant negative inhibition of RNA replication and is required for virus assemply. HDV RNA replication proceeds by a double rolling circle mechanism of RNA-dependent RNA replication. It is probably mediated by RNA polymerase II. How pol II utilizes RNA as template is not clear. We hypothesize that cellular DNA-dependent RNA transcription factors are involved in HDV RNA replication, and thus examined the possible interaction of the known transcription factors with HDV RNA and HDAg. We show by in vitro binding studies that TATA-binding protein (TBP) binds specifically to HDV RNA, and SP1 binds to HDAg, which, in turn, binds to HDV RNA. The binding was also demonstrated in the cells. Furthermore, both TBP, SP1, pol II and HDAg are part of the ribonucleoprotein complex, even though pol II binds directly to neither HDV RNA nor HDAg. We propose that the template specificity of pol II may be expanded to RNA because of the direct and indirect interactions of transcription factors with HDV RNA and HDAg. Thus, the pol II transcription machinery is used for HDV RNA replication. We have further shown that Rb and AP1 are also part of the HDV RNP. This result suggests that sequestering of transcription factors may be a mechanism for HDV pathogenesis and HDV-induced inhibition of HBV replication.

NZ 011 REPLICATION OF HEPATITIS B VIRUSES, Christoph Seeger, Guang-Hua Wang and Fabien Zoulim, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia.

Infection and persistence of hepatitis B viruses requires the synthesis of an episomal DNA template that is generated and maintained by reverse transcription of an RNA intermediate, the pregenome. The reverse transcriptase responsible for this reaction is encoded by the viral polymerase (pol) gene. The function of this polypeptide is, however, not restricted to DNA polymerization and degradation of RNA, both of which are innate activities of other known reverse transcriptases. It acts as a primer for reverse transcription and, as a consequence of this function, becomes covalently linked to the 5' end of the reverse transcribed DNA strand. While this priming reaction is reminiscent of the mechanism described for the initiation of DNA synthesis of double stranded DNA viruses, including adenovirus and the bacteriophage phi-29, the molecular details are actually novel. For example, priming and DNA polymerization activities reside on the same polypeptide and do not depend on a terminal protein. Moreover, DNA synthesis is not entirely processive. Reverse transcription initiates near the 5' end of the viral RNA at an RNA hairpin, the presumptive binding site for the polymerase. The binding of the polymerase to the RNA hairpin is critical for the activation of the polymerase to prime DNA synthesis. Moreover, it is required for the packaging of pregenomic RNA into subviral (core) particles. A bulge in the hairpin provides the template for the synthesis of a short DNA strand that is covalently linked to a tyrosine residue of the polymerase polypeptide. Subsequently, the DNA protein complex is translocated to the 3' end of the viral RNA where minus strand DNA can resume and proceed continuously to the 5' end of viral RNA.

Genetic Instability I (Joint)

NZ 012 P53 AND OTHER MOLECULAR CONTROLS OF THE RESPONSE TO DNA DAMAGE, Michael B. Kastan, William G. Nelson, Chaw-Yuan Chen, and William B. Slichenmyer, The Johns Hopkins Oncology Center, Baltimore, Md. 21287.

A variety of cellular responses to DNA damage influence cellular fate, such as whether heritable genetic alterations are passed on to daughter cells and whether the cell survives the damaging insult. Efficiency of repair of the damage and the timing of this repair relative to critical cellular processes, such as DNA replication or chromosome segregation, are two important parameters dictating cellular outcome. We have recently characterized a signal transduction pathway which dictates whether or not a cell will arrest in G1 following exposure of mammalian cells to various DNA damaging agents. P53, the most commonly mutated gene in human cancers characterized to date, is a critical participant in this cellular response pathway. Levels of p53 protein transiently increase by a post-transcriptional mechanism in response to DNA damage and result in a transient G1 arrest. Cells lacking normal p53 function continue to enter S-phase and replicate their DNA despite the presence of the DNA damage. The gene product(s) which is(are) defective in the cancer-prone disease, ataxia-telangiectasia (A-T), is required for optimal induction of p53 protein by ionizing radiation since cells from A-T patients are quantitatively defective in p53 induction. DNA damage-induced increases in p53 protein also result in increases in transcription of the GADD45 and MDM2 genes; the former gene product may be involved in mediating the G1 arrest and the latter may participate in a "feedback" loop which limits the length of the G1 arrest. Though exposure of cells to many different types of DNA damaging agents results in p53 induction, it appears that DNA strand breaks are sufficient and are probably a necessary intermediate signal in the DNA for initiation of this pathway. In some cell types, initiation of this pathway results in apoptotic cell death rather than in a G1 arrest; in these cells, loss of p53 function results in a relative increase in resistance to the cytotoxic effects of the DNA damage. Interestingly, however, in contrast to the consequences of loss of the G2 checkpoint in yeast when the rad9 gene is mutated, in mammalian cells in which a G1 arrest (rather than apoptosis) is the usual result of the DNA damage, no change in radiosensitivity is noted when p53 function is disrupted. Further characterization of the molecular steps in this pathway and the physiologic ramifications of dysfunction of the pathway is continuing.

NZ 013 ABROGATION OF CELL CYCLE CHECKPOINT CONTROL IN PRENEOPLASTIC CELLS, Thea D. Tlsty*†, *Lineberger Comprehensive Cancer Center, Department of Pathology, and †Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, NC 27599-7295

Genomic integrity is maintained by a network of cellular activities that assesses the status of the genome at a given point in time and provides signals to proceed with or halt cell cycle progression. Mutations in any part of these cellular pathways can have the ultimate effect of disrupting chromosomal integrity. We have used viral proteins involved in malignant transformation to investigate cellular pathways that may be perturbed during loss of genomic stability. Recent studies have identified cellular proteins which are targets for the viral oncoproteins, stressing the importance of these cellular proteins in controlling neoplasia. Among the targets of the viral oncoproteins are the products of the p53 and retinoblastoma (Rb) tumor suppressor genes. We demonstrate that the expression of human papillomavirus type16 E6 and E7 oncoproteins in normal, mortal cells disrupts the integration of the network of signals that maintain genomic integrity. E6-expressing cells, in which cellular p53 protein is bound and degraded, exhibited alterations in cell cycle control and displayed the ability to amplify the endogenous CAD gene when placed in the drug PALA. Expression of E7, which complexes with a variety of cellular proteins including Rb, resulted in an p53-independent alteration in cell cycle control, massive cell death, and polyploidy upon PALA treatment. These results demonstrate that the viral proteins disrupt cellular processes that safeguard the genome and growth of normal cells. Alterations of these controls are being examined in cells from patients that are predisposed to neoplasia.

Papillomaviruses: Transcription and Replication

NZ 014 REGULATION OF HUMAN PAPILLOMAVIRUSES IN DIFFERENTIATING EPITHELLIUM, Laimonis A. Laimins, Dept. of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

Papillomaviruses infect cutaneous and mucosal epithellium and induce a variety of proliferative lesions in most mammals including humans. Of the 66 types of human papillomaviruses viruses that have been identified, a subset, including types 16, 18, 31, 33 and 51, are associated at a high frequency with anogenital cancers. These anogenital cancers such as cervical cancer, develop from precursor lesions termed cervical intraepithelial neoplasias (CIN) which are graded from I to III depending on the disruption of the pattern of epithelial differentiation. In low grade CIN lesions, only a slight alteration of differentiation occurs and HPV DNA is found as multicopy episomes in basal cells. The production of viral particles, genome amplification, capsid protein synthesis, and virion assembly occur in a differentiation dependent manner in suprabasal cells. In carcinomas, viral DNA is usually integrated into host chromosomes, and no viral production occurs. Studies on the life cycle of human papillomavirus have been limited in the past due to an inability to duplicate the program of epithelial differentiation in vitro. Recently, my laboratory has utilized an in vitro system which allows for the stratification and differentiation of keratinocytes at the air-liquid interface of collagen raft cultures to examine the life cycle of human papillomaviruses. Using cell lines (designated CIN-612) which stably maintain episomal copies of HPV, we have been able to duplicate most aspects of the productive life cycle of HPV type, 31b. When CIN-612 cells were allowed to stratify in the raft cultures, tissue cross sections exhibited morphologies similar to CIN I lesions in vivo with the appearance of many koilocytic-like nuclei in suprabasal layers. In situ hybridization analysis of CIN612 raft cross sections demonstrated specific amplification of HPV31b DNA in the upper layers of differentiating cells similar to that seen in biopsies of low grade lesions in vivo. By modifying culture conditions through the addition of phorbol esters, we have induced the expression of capsid protein and synthesis of virions. In this system, HPV31b transcripts have been detected which are expressed constitutively throughout the stratified epithelium as well as a set of transcripts that are induced upon differentiation. This later class include transcripts for E1^E4 and late capsid genes L1 and L2 which initiate from novel-differentiation dependent promoters present in the E7 and E1 genes. The role of viral and cellular factors in the regulation of the differentiation-dependent processes of transcription and replication will be discussed.

NZ 015 FUNCTIONAL ANALYSIS OF THE PAPILLOMAVIRUS REGULATORY PROTEINS **Alison A. McBride[1], Patricia Winokur[2], Trina Sarafi[3] and Marc Carruth*,** [1] National Cancer Institute, Bethesda, Maryland, [2] Iowa University Hospital, Iowa, [3] Washington University, St Louis, * HHMI-NIH Research Scholar

The papillomavirus E1 and E2 proteins are essential for viral DNA replication and play a pivotal role in viral transcriptional regulation.The bovine papillomavirus type 1 (BPV-1) encodes for three E2 proteins. The E2 transcriptional transactivator consists of a large N-terminal transactivation domain linked to a C-terminal DNA binding/dimerization domain. Two transcriptional repressors are encoded by the 3' end of the E2 open reading frame and contain only the DNA binding/dimerization domain. Analysis of E2 mutants that separate the DNA binding and dimerization functions of the E2 protein indicate that the E2 repressors can function both by competition for DNA binding and by the formation of heterodimers with the transactivator protein. E2 DNA binding can also be regulated, at least *in vitro*, by oxidation-reduction of a conserved cysteine in the DNA binding domain.

The E2 transactivator is required for viral DNA replication (1). In general, an intact transactivation and DNA binding domain are required for this function and E2 mutants have been identified that separate the transactivation and replication properties of the protein. The E2 transactivator binds to the replication origin in complex with the viral E1 protein (2). Regions of the E2 protein important for this interaction have been identified. The major E2 phosphorylation sites have been identified and E2 phosphorylation may regulate viral copy number.

The E1 protein has several replication-associated properties such as origin-specific binding and helicase activities. The regions of the E1 protein required for origin specific binding, co-operative binding with the E2 protein and interaction with the E2 protein have been identified.

1. Ustav and Stenlund (1991) EMBO J. 10:449. 2. Mohr et al., (1990) Science 250:1694.

NZ 016 DNA REPLICATION OF PAPILLOMAVIRUSES, Juhan Sedman[1], Mart Ustav[2] and Arne Stenlund[1], [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, [2] Estonian Biocenter, Tartu, Estonia.

Replication of the papillomavirus genome in vivo requires two viral proteins encoded from the E1 and E2 open reading frames. The necessary cis-acting sequences (Ori) include specific binding sites for both of these proteins. The position and distance of the E2 binding site relative to the E1 binding site is not critical for replication activity and the Ori therefore appears to have a bi-partite structure. Replication of bovine papillomavirus (BPV) has also been studied using a cell free replication system. This system shows no requirement for either the E2 protein or for the presence of an E2 binding site at the Ori. We have used E1 and E2 proteins purified from E. coli for in vitro binding studies. Gel shift assays and DNAse footprint analysis both demonstrate that cooperative binding of E1 and E2 to the Ori requires binding sites for both proteins. The Ori complex formed by E1 alone shows distinctive differences compared to the complex formed in the presence of both E1 and E2. Interference analysis and hydroxy-radical footprints demonstrate that the recognition sequence for E1 at the Ori is different in the presence of E2, furthermore, the number of E1 molecules in the two complexes differs, demonstrating that E2 has a qualitative effect on complex formation. Analysis of a variety of Ori mutants demonstrate a perfect correlation between the ability of a specific construct to replicate in vivo and its ability to form the specific E1+E2/ Ori complex in vitro. This correlation does not extend to replication in a cell free system which shows no dependence on E2. Based on these observations we propose that for replication in vivo, the E2 induced Ori complex is specifically required, while the E1/Ori complex represents a different pathway.

An interesting aspect of the viral life cycle is the capacity of the viral genome to persist as a multicopy plasmid in infected cells and to be faithfully inherited by the daughter cells at cell division. This process requires specific mechanisms in addition to DNA replication such as systems for control of partitioning and copy number. We have identified a sequence in the BPV genome as a candidate for a partitioning function. This sequence is required in addition to the minimal Ori for stable maintenance of BPV. In addition, this sequence can function to increase the transformation eficiency of non-replicating plasmids in an E1 and E2 dependent manner. These results indicate that the element may function through a mechanism that involves preferrential nuclear retention.

Human Retroviruses

NZ 017 DEVELOPMENT OF RIBOZYME GENE THERAPY AGAINST HIV Flossie Wong-Staal, Mang Yu, Osamu Yamada, Mark Leavitt, Midori Maruyama, Anthony Ho et al. Departments of Biology and Medicine, UCSD, La Jolla, CA 92093-0665

Ribozymes are RNA molecules that contain anti-sense sequences for specific recognition, and RNA-cleaving enzymatic activity. We reported that a hairpin ribozyme designed to cleave HIV-1 RNA in the 5' leader sequence suppressed virus expression in Hela cells co-transfected with proviral DNA from diverse HIV-1 strains. Moreover, the antiviral effect was primarily due to the catalytic rather than antisense property of the ribozyme. Human $CD4^+$ T cell lines (Jurkat and Molt 4/8) transduced by murine retroviral vectors carrying the ribozyme gene persistently expressed the ribozyme gene with no apparent deleterious effect on cell proliferation or long term viability. These cells were resistant to challenge from diverse strains of HIV-1, including an uncloned clinical isolate. Furthermore, we showed that the ribozymes inhibited both early and late steps in the replication cycle of retroviruses, presumably by cleaving incoming virion RNA as well as the transcribed genomic and subgenomic mRNAs. Our more recent efforts focused on transduction of primary lymphocytes and hematopoietic progenitor cells, as these would be the realistic targets for gene therapy for AIDS patients. Primary PBL selected in culture after transdcution with the ribozyme vector also completely resisted infection by HIV-1. High transduction efficiency was obtained with enriched CD34+ cells pre-stimulated with a variety of growth factors. Persistent expression of the ribozyme was also detected in the colonies established from these progenitor cells. Experiments are in progress to determine if ribozyme transduced progenitor cells will yield progeny cells that are resistant to HIV-1 infection.

NZ 018 INTERACTION OF HTLV-1 ONCOPROTEIN WITH TRANSCRIPTION FACTORS, Mitsuaki Yoshida, Takeshi Suzuki, Junichi Fujisawa and Hiroshi Hirai, Department of Cellular and Molecular Biology, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan

A human retrovirus, HTLV-1, causes adult T-cell leukemia (ATL) and also associated with tropical spastic paraparesis (HAM/TSP) and uveitis. Persistent infection and replication of HTLV-1 seem to be crucial for the pathogenesis, since patients with all these diseases are sero-positive and have a long incubation time for the diseases. The viral regulatory gene *tax* immortalizes normal human T cells in interleukin-2 dependent fashion, transforms rodent fibroblasts in anchorage independent manner and induces solid tumors in its transgenic mice, indicating its roles in development of ATL. Function of Tax is established as transcriptional activator of the viral genome and also of certain cellular genes including those for lymphokines, lymphokine receptors, and proto-oncogenes.Tax activation of cellular genes has been proposed to ultimately contribute to the viral pathogenesis inducing abnormal proliferation of infected T cells and producing various cytokines.

Tax requires enhancer for transcriptional activation and three enhancers were identified: these are 21-bp enhancer of HTLV-1, NF-kB binding site of the IL-2Ra and SRE of the c-*fos* genes. However, Tax does not bind to these enhancer DNAs directly, but proposed to associate with enhancer DNAs cooperating with cellular DNA binding proteins. Such indirect binding of Tax to DNA was demonstrated by DNA-affinity precipitation assay only in the presence of nuclear factors. Using gel-shift assay, DNA-affinity precipitation analysis, and immunoprecipitation, these nuclear factors, which required for Tax binding to DNA, were identified. These are CREB (CRE binding protein) and CREM (CRE modulator protein) for 21-bp enhancer, NF-kB p50 and its precursor p105 for NF-kB binding site, and SRF (serum response factor) for the SRE. Therefore, Tax can associate with enhancer binding proteins which are specific to each enhancer required for Tax trans-activation and suggested to activate the transcriptional initiation. These conclusion was further supported by the binding abilities of Tax mutants which are active or inactive in trans-activation.

In addition to the DNA association of Tax, Tax also contribute for translocation of NF-kB proteins. Additional targets for Tax binding, and their roles in transcriptional activation and pathogenesis will be discussed.

Viral Immunity and Pathogenesis

NZ 019 DESIGNING ANTI-VIRAL SYNTHETIC PEPTIDE VACCINES, Jay A. Berzofsky, Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, NIH, Bethesda, MD 20892

Synthetic peptide vaccines have the advantages that they can be designed to contain only the desired antigenic determinants for specific responses without other determinants that might elicit unwanted responses, and also that the determinants themselves can be modified to enhance potency or breadth of crossreactivity. However, they can have the disadvantage that any single determinant may be presented by only a limited selection of major histocompatibility complex (MHC) molecules of the species, and therefore would be useful in only a subset of the population. We have approached all of these issues in attempting to develop a synthetic HIV vaccine. To overcome the problem of MHC polymorphism, we have tried to identify determinants that are presented by multiple MHC molecules, and have also located multideterminant regions of the HIV-1 envelope protein that contain overlapping determinants each presented by different class II MHC molecules, so that the whole multideterminant region is presented by multiple MHC molecules. Peptides encompassing such a multideterminant region, called cluster peptides because they span a cluster of overlapping determinants, were found to be recognized by murine helper T cells of 4 MHC types and human helper T cells of diverse HLA types. These cluster peptides have been used as a source of help in the synthetic vaccine constructs. As a single epitope that is recognized by both neutralizing antibodies and cytotoxic T lymphocytes (CTL) of both mice and humans, we have used a 15-residue segment of the V3 loop of HIV-1 gp160, called P18. Constructs containing both a cluster peptide and P18 elicit very high titers of neutralizing antibodies (90% neutralization at 1:2000 to 1:16,000) in mice of several MHC types, and also $CD8^+$ CTL specific for P18 and capable of killing targets endogenously expressing the whole HIV-1 envelope protein gp160. In contrast, P18 alone hardly elicits either of these responses. Thus, the helper sites are necessary to make P18 immunogenic for eliciting either neutralizing antibodies or CTL. These vaccine constructs are potent and also avoid sites on gp160 that are known to elicit enhancing antibodies or autoimmune responses that might contribute to disease pathogenesis. However, we can potentially improve on these by tinkering with the internal structure of the individual epitopes. We have found that replacing a negatively charged glutamic acid residue with an uncharged amino acid in one of the helper determinants makes it 10 to 100-fold more potent in binding to the class II MHC molecule and in eliciting murine helper T cells that still recognize the natural HIV-1 sequence. Thus, such a modified peptide should be more potent as a vaccine, while retaining the ability to elicit T cells that will respond to HIV proteins that of course do not have the altered sequence. Similarly, we have found that making a chimeric peptide from P18, using a key residue from the sequence of the HIV-1 IIIB strain inserted in the sequence from the HIV-1 MN strain, allows us to induce in mice much more broadly crossreactive CTL that recognize sequences not only from both of these strains but from others as well. Thus, by learning how these peptides bind to MHC molecules and T-cell receptors, we can design internally modified determinants to construct more potent or more crossreactive second generation vaccines.

NZ 020 SELF-ASSEMBLED PAPILLOMAVIRUS STRUCTURAL PROTEINS: POTENTIAL APPLICATION TO VIRAL BIOLOGY, SCREENING ASSAYS, AND VACCINATION, Douglas R. Lowy[1], Reinhard Kirnbauer[1], Nancy L. Hubbert[1], Janet Taub[1], Heather Greenstone[1], Richard Roden[1], Thomas M. Becker[2], Cosette M. Wheeler[2], and John T. Schiller[1], [1]Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD 20892, and [2]University of New Mexico, Albuquerque, NM 87131.

Genital infection with human papillomavirus (HPV), most often HPV16, is the most significant risk factor for the development of cervical cancer. To have a source of properly folded capsid proteins that might be used for developing a serologic assay for HPV infection and for testing as a vaccine to prevent HPV infection, we have employed insect cells to produce the papillomavirus L1 major capsid protein. When the HPV16-L1 gene derived from a condyloma was inserted into a baculovirus vector and used to infect insect cells, preparative amounts of self-assembled HPV16-L1 particles that were indistinguishable from HPV virions were obtained. In contrast, L1 protein from the widely used HPV16 prototype strain, which we find contains a single amino acid change, did not assemble efficiently. The virus-like particles also incorporated the L2 minor capsid protein, when it is expressed simultaneously with L1, with the efficiency of L1/L2 particle formation being greater than that of L1 particles. Using the HPV16 virus-like particles, we have developed an ELISA and examined sera of 122 women with known genital HPV status. Two-thirds of women positive for HPV16 DNA were positive in the ELISA, whereas less than 10% of women negative for HPV infection or positive for infection with low risk HPV types 6 or 11 were ELISA positive. Eighty-two percent of HPV16 positive women with cervical atypia or mild dysplasia were positive. Only 53% of women positive for HPV16 by PCR but with normal Pap smears were positive. The serum antibodies recognize conformation dependent epitopes present in the self-assembled particles, in that the positive sera were negative when non-assembled viral protein was used. Analogous L1 particles from bovine papillomavirus induced high levels of neutralizing antibodies. The results suggest it may be possible to develop an ELISA to detect clinically significant "high-risk" HPV infection and that the virus-like particles might have potential as a vaccine to prevent HPV infection.

Late Breaking News II (Joint)

NZ 021 THE USE OF INTERFERONS TO RESCUE TWO NOVEL DEATH ASSOCIATED GENES AND TO IDENTIFY MOLECULAR MECHANISMS OF CELL CYCLE ARREST, Adi Kimchi[1], Dror Melamed[1], Naomi Levy[1], Lena Feinstein[1], Louis Deiss[2], Hanna Berissi[1], Tal Raveh[1], Nava Tiefenbrun[1] and Ofer Cohen[1], [1] Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot 76100, [2] The George Williams Hooper Foundation,University of California, San Francisco, California.

Interferons (IFNs) and IL-6 are potent growth inhibitory cytokines that induce cell cycle arrest at the G0/G1 phase in a variety of hematopoietic and epithelial cells. The molecular mechanisms through which the cytokine-receptor interactions modify the expression or the function of few cell cycle controlling genes were analysed in order to elucidate the genetic control of proliferation arrest in mammalian cells (reviewed in 1).Two of the recently studied downstream target genes will be discussed: E2F and wt p53. We found that IFNs (α or β) or IL-6 suppressed within few hours the DNA-binding activity of E2F in hematopoietic cells. The inhibitory effect could be reversed by the removal of Mg^{2+} cations from the cell extracts suggesting that the cytokines either modified E2F post-translationally or induced/activated a putative DNA-binding inhibitor (2). This novel type of E2F regulation by IFNs and IL-6 contributed to the cytokine-induced c-*myc* suppression. Single and double transfections with wt p53 and deregulated *c-myc* proved the existence of complementary interactions between the outcome of p53 activation and of *c-myc* suppression in the control of the cytokine-triggered cell cycle arrest (3).
In some cells IFN-γ also induces programmed cell death that is characterized by distinct cytological features.We have employed a method of anti-sense selection of genes (4) to rescue cDNAs that mediate this type of cell death. The cells were transfected with an anti-sense cDNA expression library, cloned in EBV-based vector and episomal DNA was rescued from cells that survived in the presence of IFN-γ. Two novel anti-sense cDNA clones were isolated, each suppressing the occurence of the death associated morphological alterations in the continuous presence of the cytokine. One, designated DAP-1, is expressed as a single 2.4 Kb mRNA transcript which codes for a 15kDal basic protein that carries the RGD motif and few potential phosphorylation sites. The second, is transcribed into a single 6.3 Kb mRNA which has the potential to code for a unique calmodulin-dependent serine/threonine kinase (termed DAP-kinase) that carries 8 ankyrin repeats . The two genes are widely expressed in a variety of cells and may represent novel cell death associated genes.

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Retroviruses, Hepatotropic Viruses, and Cellular Proliferation

NZ 100 PRODUCTION OF IL-1ß AND CD23 BY HUMAN B LYMPHOCYTES: COOPERATIVE EFFECTS OF HIV-1 AND EBV, R. Berger and J.D. Schwarzmeier, Ludwig-Boltzmann-Institute for Cytokine Research, Vienna, Austria

B cell lymphomas are frequently occurring in patients infected with the human immunodeficiency virus type 1 (HIV-1). To assess a possible role of HIV-1 in the activation of B cells and to identify critical elements in B cell proliferation, we infected primary human B cells from HIV-1-seronegative, Epstein-Barr virus (EBV)-seropositive donors by cell-free culture supernatants of HIV-1 strain 3B. 48 hours after infection HIV-1-specific mRNA was detectable by Northern blotting and virus capsid protein (p24) was secreted 4 days after onset of cultures. A profound proliferative response as revealed by ^{3}H-thymidine incorporation was also seen in these cultures. In a second set of experiments, recombinant HIV-1 proteins were used instead of infectous virus. While the addition of gp120, p24, p55, rev or nef had no effects, the transactivating protein tat led to a significant uptake of ^{3}H-tymidine. Moreover, tat-stimulated B cells continued to proliferate and finally resulted in EBV-positive B cell lines. To analyze the production of cytokines relevant for B cell growth, ELISA's specific for IL-1ß and sCD23 were used. Kinetic studies revealed that immediately after infection none of these cytokines were produced. After 2 weeks, however, high amounts of IL-1ß and sCD23 could be detected in culture supernatants and paralleled the occurrence of an EBV-encoded antigen (EBNA-2), known to upregulate CD23-expression in B cells. Both cytokines are induced by EBV rather than by HIV-1, since transfection of B cells with plasmids encoding the EBNA-2 gene efficently induced CD23 as well as IL-1ß secretion in B cells, while tat-encoding plasmids failed to do so.

Based on our results it is tempting to speculate, that HIV-1/tat activates endogenous EBV and that B cell proliferation seen in AIDS is mediated via cooperative effects between HIV-1 and EBV leading to the secretion of cytokines important for B cell growth.

NZ 101 ANALYSIS OF THE p53 TUMOR SUPPRESSOR PROTEIN IN HTLV-I/II-INFECTED CELL LINES Charlene S. Dezzutti, Renu B. Lal, and Michael D. Lairmore* Retrovirus Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333 and *Center for Retrovirus Research & Department of Veterinary Pathobiology, The Ohio State University, Columbus, OH 43210

Human T-lymphotropic virus type I (HTLV-I) has been etiologically linked to several diseases including adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy (HAM). A closely related retrovirus, HTLV-II, has not been conclusively linked to any specific disease. Both viruses are able to transform lymphocytes *in vitro*. In order to better understand the transformation process, we examined for the presence of the p53 in 13 HTLV-I-infected cell lines (3 HAM-derived, 6 ATL-derived, and 4 asymptomatic-derived cell lines), and 5 HTLV-II-infected cell lines (all derived from asymptomatic persons). Three monoclonal antibodies (mAb) (DO-1, PAb 421, and PAb 240) were used to detect the presence of wild type or mutant p53. All 3 mAb detect both wild type and mutant forms of p53 by Western blot; however, mAb 240 only detects mutant p53 by radioimmunoprecipitation (RIP). By Western blot, all of the cell lines examined had detectable p53 using the PAb 421 and PAb 240 mAb. Interestingly, the DO-1 mAb detected doublet p53 bands in 10 of 13 HTLV-I-infected cell lines. Only 1 of 5 the HTLV-II-infected cell lines contained the doublet p53 form. Preliminary RIP data of p53 from these cell lines indicate that while mAb DO-1 and PAb 421 detect the wild type form of p53 in the cell lines tested to date, none have reacted with PAb 240 which detects the mutant form of p53 in RIP. With the sensitivity of the mAb PAb 240, these data imply that p53 does not have this mutation in HTLV-I- or HTLV-II-infected cell lines derived from ATL or HAM patients or asymptomatic persons. The significance of the doublet p53 bands, an indication of altered phosphorylation, in transformation is being examined.

NZ 102 MOLECULAR CHARACTERIZATION OF HTLV-I T-CELL LYMPHOMAS IN SCID MICE, Gerold Feuer, Sheila Stewart, Jerome A. Zack, Fred Lee, Ralph Feuer and Irvin S.Y. Chen, Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024-1678

Events resulting in tumorigenesis following human T-cell leukemia virus (HTLV-I) infection remain obscure. To explore the possibility of developing a small animal model to study HTLV infected human cells *in vivo*, severe combined immunodeficient (SCID) mice were injected intraperitoneally with peripheral blood lymphocytes (PBL) from patients diagnosed with Adult T-cell leukemia (ATL). Many of these mice became persistently infected with HTLV-I and lymphoblastic lymphomas developed in mice injected with PBL from two ATL patients. Tumor cells recovered from SCID mice resembled ATL cells from patients, with respect to clonality and in the expression of the CD4 and CD25 (IL-2R) cell surface markers. Extensive deletions of the HTLV-I proviral sequences were detected in tumors that developed in SCID mice, although the tax/rex sequences were invariably maintained. This system represents the first animal model to study HTLV-I tumorigenesis *in vivo*.

In contrast to ATL tumor cells, cell lines derived by HTLV immortalization of T-cells *in vitro* do not persist or form tumors following inoculation into SCID mice. Although SCID mice are immunocompromised, they retain functional natural killer (NK) cells and macrophages and are capable of rejecting xenografts. Immunosuppression of SCID mice, either by sublethal whole body irradiation prior to inoculation of T-cell lines, or by concurrent injection of anti-asialo GM_1 (an antiserum which transiently reduces NK activity *in vivo*) allows some HTLV-I infected T-cell lines to engraft and form tumors. Biological differences between the ATL tumor cells established in SCID mice from patients and T-cell lines transformed by HTLV *in vitro*, and their respective abilities to engraft in SCID mice will be presented.

NZ 103 ON THE SIGNIFICANCE CONSERVED RESIDUES IN THE $P6^{GAG}$ REGION OF HIV-1 $PR55^{GAG}$, Robert J. Gorelick, D.E. Ott, R.C. Sowder, II, S.M. Nigida, Jr., L.E. Henderson, and L.O. Arthur. AIDS Vaccine Program, PRI/DynCorp, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

HIV-1(MN)-infected H9 cells were cloned by limiting dilution and 124 individual clones were obtained. These were assayed and three clones produced high levels of infectious virus. Southern blot analysis of DNA from these high producer clones revealed the presence of at least two proviral sequences in each clone. One of these high producer clones, designated H9/HIV-1(MN) Clone 4, was further characterized. HPLC analysis of the Gag cleavage products from sucrose-banded purified Clone 4 virus particles detected two forms of the $p1^{Gag}$ and $p6^{Gag}$ peptides. Protein and PCR sequence analysis was used to determine the structure of these cleavage products. One provirus had protein and DNA sequences consistent with reported HIV-1(MN) sequences while the variant provirus had a duplication of 12 codons in the 3' end of the *gag* ORF coding for the $p6^{Gag}$ protein and an amino acid substitution in the $p1^{Gag}$ (Ott, unpublished results). Interestingly, nearly identical modifications were previously found in both the LAI and HX10 molecular clones of HIV-1. This observation suggests that selection pressures may contribute to the occurrence of these amino acid duplications in the $p6^{Gag}$. The duplicated region includes a conserved PTAPP amino acid motif (found in HIV-1 and other lentiviruses) and its biological significance was investigated by site-directed mutational analysis. Results show that individual residues in the PTAPP sequence are required for efficient RNA packaging but not for budding or infectivity. This suggests that the PTAPP region of $p6^{Gag}$ functions at the precursor level during viral assembly. We speculate that Gag precursors from two proviruses (one with a duplication in the $p6^{Gag}$ region) in the same cell may function in combination to increase the production of HIV-1 *in vitro* and *in vivo*.

NZ 104 CYTOTOXIC T LYMPHOCYTE MEDIATED REGULATION OF HEPATITIS B VIRUS GENE EXPRESSION IN VIVO, Luca G. Guidotti, Kazuki Ando, Monte V. Hobbs, Tetsuya Ishikawa, Laura Runkel and Francis V. Chisari, The Scripps Research Institute, La Jolla, CA 92037, USA.

During hepatitis B virus (HBV) infection, distinct host-virus interactions may establish the patterns of viral clearance and persistence, and the extent of virus-associated pathology. It is generally thought that HBV-specific class I-restricted cytotoxic T lymphocytes (CTL) play a critical role in this process by destroying infected hepatocytes, although recent evidence suggests that viral clearance can be mediated by CTL-induced noncytolytic mechanisms as well. To determine whether hepatocytolytic and/or noncytolytic mechanisms contribute to viral clearance in this infection, we injected an MHC class I restricted, CD8-positive hepatitis B surface antigen (HBsAg) specific CTL clones that express interferon gamma ($IFN\gamma$) and tumor necrosis factor alpha ($TNF\alpha$) after antigen stimulation in vitro and in vivo, in two different lineages of HBsAg transgenic mice. Both lineages exhibit constitutive production of HBsAg by most liver cells, but each differs markedly in the extent of hepatocyte destruction following adoptive transfer of $1x10^7$ CTL clones. In the current study we report that administration of HBsAg-specific CTL clones results in a profound suppression of hepatocellular HBV gene expression in both lineages, thus indicating that CTL-mediated cytotoxicity is not the sole mechanism for viral gene regulation. By analyzing CTL-infiltrated livers for the presence of cytokine mRNA, we found early increases in the transcripts levels for interleukin 1 alpha ($IL1\alpha$), $IL1\beta$, $TNF\alpha$ and $IFN\gamma$, the latter two of which are signature cytokines of the CTL clones. Using neutralizing anti-cytokine antibodies, we also show that the regulatory effect of the CTL is initially mediated by $IFN\gamma$ and $TNF\alpha$, but it is delayed in onset relative to the induction of these cytokines in the liver, and it becomes independent of these cytokines shortly after it begins. The data indicate that the class I restricted CTL response activates a complex regulatory cascade within the host that inhibits hepatocellular HBV gene expression without killing the cell.

NZ 105 CELLULAR PROTEINS ASSOCIATED WITH HIV-1 AND SIV VIRIONS, Louis E. Henderson, R.C. Sowder, II, D.G. Johnson, L.V. Coren, D.E. Ott, J.W. Bess, Jr. and L.O. Arthur. AIDS Vaccine Program, PRI/DynCorp, NCI-FCRDC, Frederick, MD 21702-1201

Recently, we showed that cellular proteins derived from the major histocompatibility complex (HLA class I and class II) were physically associated with HIV-1 and SIV virions produced from human T-cell lines (H9 or HuT 78 cells) (L.O. Arthur, et al., Science, 1993). In addition, we have shown that monkeys immunized with purified human HLA class II DR4 proteins are protected against a challenge dose of infectious SIV grown in HuT 78 cells (L.O. Arthur, et al., 1993, submitted). These data leave little doubt that the infectious pathogenic virion is composed of both cellular and viral proteins. In order to better understand the contributions of cellular proteins to the complete virion, it is important to know how many cellular proteins might be virion-associated and to identify as many as possible. To address these fundamental questions, we have separated proteins in viral preparations by combinations of reversed phase high pressure liquid chromatography followed by SDS-PAGE and determined their amino acid sequences by direct automated Edman degradations. Proteins were identified by comparing their determined amino acid sequences to known sequences in computerized protein and nucleic acid data bases. At present, we have compiled a list of over 40 cellular proteins, termed copurifying cellular proteins (CCP) that are found in highly purified (sucrose density gradient banded) viral preparations. The list of CCP is dependent upon the virus (HIV-1 or SIV) and upon the cell line used to propagate the virus (H9, HuT 78 or CEM) and some CCP are common to all preparations. Where possible, we have prepared or obtained specific antisera to the proteins on the CCP list and used immunoblot assays for their detection and developed specific radioimmune competitions assays for their quantitation. To determine whether or not a specific protein on the CCP list is physically associated with the virion particle, we are using analytical procedures that include immune precipitation of whole virion particles, chemical cross linking and partial proteolysis. These results and the CCP lists will be presented. The results strongly support the proposition that the mature virions are composed of both cellular and viral proteins.

NZ 106 OVERPRODUCTION OF NFKB2 AND c-REL: A MECHANISM FOR HTLV-I TAX-MEDIATED *trans*-ACTIVATION VIA THE NF-κB SIGNALLING PATHWAY.

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Molecular, biochemical and epidemiological evidence implicate HTLV-I as an etiologic agent of adult T cell leukemia (ATL). The Tax protein of HTLV-I, a positive transcriptional activator of HTLV-I gene expression, is a viral oncogene that also increases transcription of cellular genes including GM-CSF, IL-2Rα and IL-2. One of the cellular targets of the *trans*-activating effects of Tax is the NF-κB/Rel family of transcription factors, pleiotropic regulators of immunoregulatory, cytokine and viral gene expression. In this report, we demonstrate that NFKB2 and c-Rel are overexpressed in HTLV-I infected and Tax-expressing cells and, together, account for the majority of the constitutive NF-κB binding activity in these cells before and after PMA stimulation. Most importantly, we show a Tax-dependent correlation between expression of NFKB2(p100) and processing to the DNA binding NFKB2(p52) form, induction of c-Rel, and *trans*-activation of NF-κB-mediated gene expression. Furthermore, the NFKB2 precursor is physically associated with c-Rel and with Tax in HTLV-I infected cells. We propose that NFKB2 synthesis and processing allows continuous nuclear expression of an otherwise cytoplasmic protein and, in conjunction with overexpression of c-Rel, NFKB2 alters the NF-κB signalling pathway and contributes to leukemic transformation of T cells by HTLV-I.

NZ 107 IDENTIFICATION OF BIOLOGICALLY ACTIVE HUMAN ENDOGENOUS RETROVIRUSES (HERV), Reinhard Kurth, Roswitha Löwer, Klaus Boller, Brigitte Hasenmaier, Christine Korbmacher, Joachim Denner, Ralph Tönjes, Christiane Limbach, Raimond Lugert, Johannes Löwer, Paul-Ehrlich-Institut, 63225 Langen, Germany

Retroviruses comprise strains with considerable disease potential. Endogenous virus strains can be pathogenic in rodents and may also be considered as mobile genetic elements with the potential to produce mutations. Employing a generally applicable method for the detection of even unknown intracellular retroviral RNA (Virology 192, 501, 1993) we have identified HERV-K (K for $tRNA_{Lys}$ used as primer) whose mRNA expression pattern is reminiscent of that seen with complex exogenous strains (PNAS 90, 4480, 1993). HERV-K code for the "Human Teratocarcinoma-Derived Retroviruses (HTDV)" previously identified by electron microscopy (Virology 196, 349, 1993). All HERV-K genes have been cloned and sequenced and shown to contain long open reading frames. Although there is as yet no genetic evidence that HERV-K is defective, we could not yet transmit the virus to novel host cells. We are presently investigating the intracellular regulation of HERV-K transcription and a possible association of HERV-K expression with human diseases.

NZ 108 IDENTIFICATION OF AN HBV X-ASSOCIATED PROTEIN AS A PROBABLE DNA-REPAIR PROTEIN, Teh-Hsiu Lee, Stephen J. Elledge,* and Janet S. Butel, Division of Molecular Virology and *Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030

The hepatitis B virus X protein is known to transactivate several viral and cellular genes. However, the mode of transactivation remains unclear. We employed the yeast two-hybrid system to search for HBV X-associated proteins. The X open reading frame was subcloned in-frame into a plasmid encoding the DNA-binding domain of the yeast Gal4 protein. A library containing cDNA from Epstein-Barr virus-immortalized human lymphocytes fused to the activation domain of the Gal4 protein was constructed. Yeast strain Y153, carrying the LacZ and His3 genes controlled by a Gal4-regulated promoter, was used as the host for co-transformation. An interaction between X and any X-associated protein would bring the Gal4 DNA-binding and activation domains together and activate the expression of LacZ and His3 genes, which can be detected by X-gal staining and growth in his-deficient medium. From $\sim 1 \times 10^6$ yeast clones harboring both plasmids, we isolated 67 positive clones encoding putative X-interactive proteins. False-positive clones were eliminated by showing an interaction with unrelated (non-X) genes. One clone, designated XAP-1, was found to be X-specific. XAP-1 is the human homologue of the monkey UV-DDB gene; the UV-DDB protein is defective in some xeroderma pigmentosum group E patients. The interaction between HBV X and XAP-1 was confirmed by specific binding of XAP-1 translated *in vitro* to X protein expressed as a GST (glutathione-S-transferase) fusion protein. The possible significance of this interaction in HBV replication, transcription and carcinogenesis will be discussed.

NZ 109 ACTIVATION OF THE TRANSCRIPTION FACTOR C-JUN/AP1 BY THE HEPATITIS B VIRUS TRANSACTIVATOR PX OCCURS THROUGH A RAS- AND RAF-DEPENDENT SIGNALLING PATHWAY.

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The Hepatitis B Virus X protein is a promiscuous transactivator of RNA polymerase II as well as RNA polymerase III promoters, whose mechanisms of action are poorly understood. Previous reports suggested the possibility of a direct interaction between pX, which is devoid of DNA binding activity, and components of the cellular transcription machinery (such as CREB/ATF2), and it was shown that pX might indeed possess an acidic domain that makes it able to activate transcription when fused to the DNA binding domain of the liver restricted transcription factor C/EBP. Recently, to better explain the promiscuity of pX activity on transcription, alternative models, based on the activation of cellular kinases involved in growth regulation and control, have been proposed. We investigated the mechanisms of c-Jun activation by pX and in particular the role of cellular proteins involved in signal transduction (namely Ha-Ras and Raf-1). In both HeLa and undifferentiated F9 cells pX was able to increase the activity of exogenous transfected wild-type c-Jun, but not of c-Jun mutants bearing mutations in the amino-terminal serine residues. We show, by use of HaRas and Raf-1 dominant negative mutants that both HaRas and Raf1 are required for pX-induced activation of c-jun transcriptional activity. In addition we show that pX is able to cooperate with Raf-1 in c-Jun activation. Our results are consistent with the hypothesis that at least one site of action of pX is peripheral and is located upstream of the Ras genes products.

NZ 110 AMINO-TERMINAL DOMAIN OF ATF3 MEDIATES HTLV-1 TAX-DEPENDENT TRANSACTIVATION OF GENE TRANSCRIPTION, Kenneth G. Low, Hung-Ming Chu, Yi Tan, Phillip M. Schwartz, Gwynn M. Daniels‡, Michael H. Melner† and Michael J. Comb, Laboratory of Molecular Neurobiology, Massachusetts General Hospital, Charlestown, MA 02129, ‡Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, OR 97201, and †Departments of Obstetrics and Gynecology, Vanderbilt University, Nashville, TN 37232

The transactivation of viral and cellular genes by the HTLV-1 Tax protein is dependent upon interactions with various cellular transcription factor proteins such as NF κB, SRF, CREB and ATF3. In order to identify domains of ATF3 which interact with Tax and confer Tax-responsiveness, the heterologous bacterial Gal4 DNA-binding domain was fused to various domains of ATF3. A chimeric Gal4 protein containing the amino-terminal 66 amino acids of ATF3 confered Tax-responsiveness to a Gal4-responsive promoter. However a chimeric Gal4 protein containing the carboxy-terminal 42 amino acids of ATF3 was unable to confer Tax-responsiveness to a Gal4-responsive promoter. In contrast, *in vitro* translated Tax specifically interacts with GST-fusion proteins containing either amino- or carboxy-terminal domains of ATF3. Furthermore, Tax-specific antisera co-immunoprecipitated *in vitro* translated ATF3 with Tax. These results implicate ATF3 in mediating Tax-dependent transactivation of gene transcription during HTLV-1 leukemogenesis.

NZ 111 DEREGULATION OF C-myb GENE EXPRESSION IN IL-2 DEPENDENT T CELL LINES IMMORTALIZED BY THE X-REGION OF HTLV-I, Kathleen L. McGuire, David B. Young, Milena Iacobelli, and Forest L. Rohwer, Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, CA 92182

The human T-cell leukemia virus type I, or HTLV-I, retrovirus is the etiologic agent of Adult T-cell leukemia (ATL). Leukemic cells resemble T cells activated by antigen in that they express Class II Major Histocompatibility Complex antigens and the α chain of the receptor for the interleukin-2 growth hormone (IL-2R), although most of these cell lines exhibit IL-2 independent growth. Several recent studies have demonstrated, however, that not all HTLV-I infected T cells are transformed. Long term culture of these non-transformed, HTLV-I infected cells requires IL-2 but they will proliferate in the absence of IL-2 to a limited extent. This limited, IL-2 independent proliferation is not blocked by the addition of cyclosporin A, an immunosuppressant which inhibits IL-2 production. However, it may be inhibited by rapamycin, a drug which inhibits T cell proliferation by blocking IL-2R signal transduction events. These data suggest that the HTLV-I retrovirus is capable of stimulating proliferation of T cells through the IL-2R pathway directly, without the need for IL-2. One essential function of IL-2/IL-2R signal transduction is the activation of c-*myb* proto-oncogene expression. This product has been demonstrated by several research groups to be activated by IL-2 in T cells and is necessary for G_1 to S transition in these cells. Therefore, it is logical to hypothesize that HTLV-I driven proliferation of T cells is the result of direct or indirect c-*myb* activation. We have demonstrated that c-*myb* expression is no longer controlled by IL-2 in dependent T cell lines immortalized to tissue culture either by HTLV-I or the X-region of this virus. In addition, our studies have shown that c-*myb* expression can not be up-regulated by IL-2 stimulation in these cells. Therefore, we are investigating further the control of c-*myb* expression in these cell lines using the immunosuppressants cyclosporin A and rapamycin. These drugs can be used to elucidate where in the pathway of T cell stimulation de-regulation of c-*myb* gene expression occurs. In addition, because *tax* is known to influence the expression of many cellular genes, we have tested the ability of this viral product to transactivate *myb* expression and the results of these ongoing studies will be presented. These studies will allow us to determine if de-regulated *myb* expression is involved in the IL-2 independent proliferation seen in HTLV-I infected but nontransformed cells, contributing to contemporary understanding of HTLV-I mediated transformation of human T cells.

NZ 112 CHARACTERIZATION OF AN EXTENDED TAX PROTEIN IN HTLV-II SUBTYPE B ISOLATES.

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We have previously reported the complete nucleotide sequence of HTLV-II_{G12}, which was originally isolated from an asymptomatic Guaymi Indian. This isolate has been demonstrated to be an HTLV-II subtype b by nucleotide sequence analysis and restriction enzyme digestion patterns. The predicted amino acid sequence of the HTLV-II_{G12} Tax protein revealed that a change of two nucleotides toward the 3' end of the *tax* sequence resulted in an Arg residue in place of the stop codon normally present in the HTLV-II_{MoT} Tax coding sequence. In vitro transcription and translation reactions provided evidence that the HTLV-II_{G12} Tax protein was larger (~40 kDa) than HTLV-II_{MoT} Tax protein (37 kDa) and approximately the same size as HTLV-I_{ATK} Tax protein (40 kDa). A synthetic peptide-based ELISA demonstrated that the HTLV-II_{G12} extended Tax protein was immunogenic and contained a highly reactive epitope. Partial sequence analysis has shown the extended Tax protein to be a characteristic feature of the majority of HTLV-II subtype b isolates and not present in any HTLV-II subtype a isolates analyzed. Characterization of the biological properties of the HTLV-II_{G12} Tax protein as compared with HTLV-II_{MoT} Tax protein are currently under way and will be presented.

NZ 113 INDUCTION OF SINGLE-STRAND DNA BREAKS IN CELLS INFECTED WITH THE HUMAN T-CELL LEUKEMIA VIRUS OR BOVINE LEUKEMIA VIRUS, Sean M. Philpott and Gertrude C. Buehring, School of Public Health, University of California at Berkeley, Berkeley, California 94720

Majone et al. (Virology 193:456, 1993) recently demonstrated that human lymphocytes transiently transfected with a plasmid encoding the $p40^{tax}$ oncoprotein of the human T-cell leukemia virus (HTLV) are twice as likely to exhibit de novo formation of micronuclei than mock transfected cells. Experiments in our laboratory have since confirmed these results, demonstrating that cells infected with HTLV-1, HTLV-2, and the closely related bovine leukemia virus (BLV) all demonstrate increased rates of micronuclei formation (Philpott and Buehring, unpublished data). These micronuclei are small nuclear bodies which result from chromosomal damage; they contain either chromosomal fragments or entire chromosomes that have failed to attach to the mitotic spindle during cell division. It could thus be suggested that HTLV $p40^{tax}$ and BLV $p38^{tax}$ oncoproteins induce cellular transformation by either inducing DNA damage or by inhibiting cellular repair of spontaneously induced damage. To test this theory, we used a fluorescence assay which detects the relative frequency of DNA breaks to examine the effects HTLV or BLV infection on the integrity of host-cell DNA. To date eighteen different cell lines have been examined; the cell lines tested were known to be either virally uninfected, or infected with various oncogenic RNA or DNA viruses. Our preliminary results suggest infection of cells with HTLV and BLV leads to a significant increase in the number of single-stranded breaks in the host-cell DNA; these changes appeared to be stable over time, suggesting they reflect an alteration in the higher-order structure of host-cell chromatin. Thus, these data support the general hypothesis that HTLV- and BLV-induced cellular transformation is related to DNA damage.

NZ 114ACTIVATION OF HIV-1 PROVIRUS BY HSV-1 INFECTION PROCEEDS THROUGH INTERACTION BETWEEN CELLULAR AND VIRAL TRANSACTIVATORS, Paula M. Pitha and Jarda Vlach, Oncology Center, The Johns Hopkins University, Baltimore, MD 21231

Molecular mechanisms of the activation of human immunodeficiency virus (HIV-1) provirus by herpes simplex virus type 1 (HSV-1) infection has been studied. Several key factors such as NF-κB-specific proteins, p55 and p85, as well as HLP-1 protein binding to the LBP-1 sequence in the HIV-1 LTR, transactivate transcription of HIV-1 LTR. The HLP-1 protein is not identical to the previously identified (BP-1 protein) expression of which is inhibited after HSV-1 infection. In cotransfection experiments, overexpression of HSV-1-encoded ICP0, but not ICP4, activates expression of HIV-1 LTR and cooperates with both the NF-κB binding protein and HIV-1-encoded transactivator, Tat. Activation by ICP0 is not associated with binding of this protein to the specific DNA element of HIV-1 LTR, however, binding of HSV-1-specific protein, TAR150, to the TAR DNA region of HIV-1 LTR was detected. This protein may represent a modified form of the HSV-1-encoded ICP4, however, its role in the activation of HIV-1 LTR is not clear. The insertion of the TAR150 binding element in front of the TK minimal promoter confers inducibility by HSV-1, but deletion of this element does not alter inducibility of HIV-1 LTR. These results suggest that the activation of HIV-1 provirus by HSV-1 is a result of a complexed interaction between viral and cellular transactivators.

NZ 115 **IDENTIFICATION OF A FUSOGENIC DOMAIN IN HTLV-II ENVELOPE**, Betty Poon, Qi-xiang Li, and Irvin S.Y. Chen, Departments of Medicine and Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024

Human T-cell leukemia virus (HTLV) I and II have differing abilities to infect and replicate in various cell lines although cell-to-cell contact is necessary for both HTLV transmissions. Studies conducted in our lab have indicated that the env gene is involved in mediating cell fusion via interactions with an as yet unidentified cellular receptor. Using a HTLV-II permissive, HTLV-I non-permissive cell line, we examined the replication and syncytia inducing capabilities of a series of full length HTLV clones that had various regions of HTLV-I env substituted into the HTLV-II genome. All recombinant clones were replication competent but only those that were capable of causing cell fusion also replicated to high titers, as assayed by p24 ELISA. Replacement of only the 5' portion of the HTLV-II env transmembrane protein, p21, with the corresponding region of HTLV-I resulted in loss of syncytium induction and infectivity. Retention of this region of HTLV II within the context of HTLV I env sequence was not sufficient to restore the phenotype but needed the additional retention of the extracellular env protein, gp46, from HTLV II. We have narrowed the necessary fusogenic domain of HTLV-II to a 64 aa. stretch in p21 that differs from HTLV-I in 13 residues. Preliminary experiments have further mapped this domain in p21 that mediates fusion to a 39 aa region that has structural similarities to a leucine zipper motif. We are presently in the process of studying expression and processing of the chimeric envelope constructs.

NZ 116 CONFORMATIONAL ASPECTS OF V3 IN HIV-1 NEUTRALIZATION: M.Reitz, M.Robert-Guroff, F.diMarzo-Veronese, A.Louie, B. Watkins, G.Gupta#, P.Lusso, and R.C.Gallo, LTCB, NCI, NIH, Bethesda MD 20892 and #Los Alamos Nation Laboratory, Los Alamos NM

The V3 loop is a principal determinant for neutralization of HIV-1 by antibodies. Antibodies to V3 have been generally considered to be sequence rather than conformation dependent and to neutralize with a rather narrow type specificity. Natural human sera frequently recognize the V3 peptide of HIV-1(MN) but not (IIIB). We have shown that many of these sera neutralize neither virus efficiently. In contrast, when the V3 of MN is substituted for the same region in IIIB, neutralization is far stronger. In some cases this appears to be due to better recognition of the MN V3 loop within the IIIB envelope framework, in some cases to the recognition of new epitopes outside of V3 in the chimeric virus but not in IIIB, and in some cases to both. In separate experiments, we have also shown that neutralization by a murine type specific antibody (M77) directed against the IIIB V3 peptide is abrogated by the introduction of a single amino acid substitution in V3, even though binding to the free peptide does not appear to be affected. The failure of M77 to neutralize a virus after a change in a non-contact residue of V3 suggests that even type specific neutralization directed against V3 at least partially conformation dependent.

NZ 117 Chromosome analyses of chronic carriers of hepatitis B virus (HBV) and molecular analyses of HBV associated primary hepatocellular carcinoma imply a mutagenic role for HBV

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Chronic infection with hepatitis B virus (HBV) is associated with 80% of the cases of primary hepatocellular carcinoma (PHC). Although HBV carriers are at a 20% lifetime risk of PHC, the role of HBV in the pathogenesis of this malignancy is still unknown. We investigated metaphase chromosome spreads of peripheral blood cells (PBCs) of 46 HBV carriers; two of whom exhibit mosaicism for supernumerary marker chromosomes. The mosaic profiles were: 46XY/47XY+mar/48XY+2mar (78/12/10 cells out of 100 analyzed); 46XY/47XY+mar (74/26 cells out of 100 analyzed). The frequency of unidentified supernumerary chromosomes (1:23) among HBV carriers is 100 times that observed among 377,357 amniocentesis samples (1:2500). Chronic carriers who are viremic (HBV DNA sero-positive) also show a significant increase in chromosome breaks in PBCs. HBV infection may initiate chromosome damage that also results in supernumerary marker mosaicism. We also performed molecular analyses of cellular DNA of the non-tumor (NT) and tumor (T) liver tissue from two cases of HBV associated PHC, without circulating HBV DNA Replicating forms of HBV DNA and random rearrangements of host genome were observed in NT tissue. In T tissue, we found integrated HBV DNA and genetic loss in the 1p region. Chronic HBV infection is associated with immune destruction of hepatocytes and regeneration of hepatic parenchyma. We hypothesize that replicating HBV in regenerating hepatocytes could accelerate the mutation rate, which would increase the probability of malignant transforming events and thereby, increase the risk of PHC.

NZ 118 ROLE OF NATURAL KILLER CELLS IN HTLV-I TUMORIGENESIS, Sheila A. Stewart*, Gerold Feuer+, Ralph Feuer+, Fred Lee+, and Irvin S. Y. Chen*+, *Microbiology and Immunology, + Division of Hematology-Oncology, Department of Medicine, Los Angeles, CA 90024-1678.

The association of HTLV-I with Adult T-cell Leukemia (ATL) has been well established while events critical to HTLV-I tumorigenesis remain to be determined. We have recently established the only small animal model that exists for HTLV-I in which transformed T cells are able to propagate in severe combined immunodeficient mice (SCID). We have previously shown that PBLs isolated from HTLV-I infected patients are able to engraft in SCID mice. In contrast HTLV-I in vitro transformed T cell lines, such as SLB-1 cells, are rapidly eliminated after inoculation into the peritoneal cavity. Immunosuppression of SCID mice with anti-asialo GM1, an antibody to a membrane ganglioside found on a variety of cell types, allows SLB-1 engraftment. SCID mice retain natural killers cells and anti-asialo GM1 transiently eliminates natural killer cell activity in vivo. We, therefore, hypothesize that natural killer cells are responsible for SLB-1 cell clearance. Presently we lack reagents to definitively study murine natural killer cells in the SCID background, therefore we are developing a RT-PCR method to detect natural killer cell activity in vivo.

Currently we are investigating which SLB-1 characteristic(s) allows natural killer cell recognition in vivo and how these differ from ATL cells which apparently avoid recognition and subsequent clearance. Analysis of SLB-1 induced tumors indicates that the tumor clone differs from the original parent cells in regards to the HTLV-I integration pattern. This suggests an in vivo selection for particular clones of SLB-1 cells with greater tumorigenic potential. These results provide a possible model for the low frequency of ATL progression observed in infected individuals. In this model natural killer cells may serve to regulate the tumorigenic potential of HTLV-I and avoidance or suppression of these cells may allow the development of ATL.

NZ 119TRANSCRIPTIONAL REGULATION OF THE HTLV-I LTR IN CELLS OF IMMUNE AND NERVOUS SYSTEM ORIGIN; Maribeth Tillmann, Renee Hinkel, and Brian Wigdahl, Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

Although human T-cell lymphotropic virus type I (HTLV-I) is an etiologic agent of adult T-cell leukemia (ATL) and tropical spastic paraparesis (TSP), the precise role of specific host and viral factors operative during the oncogenic or neuropathogenic processes associated with viral infection is currently under investigation. The result of HTLV-I infection is critically dependent on the interaction of cellular transcription factors, either alone or in conjunction with the virus-specific trans-activating protein, Tax, with the HTLV-I long terminal repeat (LTR). The HTLV-I LTR contains a Tax-inducible enhancer comprised of three imperfectly repeated 21 bp elements located within the HTLV-I LTR. Examination of the cellular factors which interact with each of the 21 bp repeats is essential to understanding the mechanisms involved in both basal and Tax-mediated transcription from the HTLV-I LTR. Utilizing electrophoretic mobility shift (EMS) analyses, we have detected 21 bp repeat-specific DNA-protein complexes when nuclear extracts derived from cells of immune and neuroglial origin were reacted with each of the three 21 bp repeat elements. Furthermore, we have also detected the presence of glial cell-specific DNA-protein complexes with isolated 21 bp repeats and with a 223 bp native fragment of the HTLV-I LTR containing all three 21 bp repeats. Characterization of the corresponding protein components indicate that members of the Sp1 and ATF/CREB families of transcription factors may participate in the formation of the 21 bp repeat-specific and glial cell-specific DNA-protein complexes, respectively. These data indicate that glial cell-specific DNA-protein complexes can form not only with isolated 21 bp repeat elements, but also with an intact native fragment of the HTLV-I LTR. The detection of cell type-dependent DNA-protein complex formation between glial cell-derived nuclear factors and the HTLV-I enhancer elements suggests the possibility that cell type-specific factors may be involved in transcriptional regulation of the HTLV-I LTR during viral infection of neuroglial elements. To examine this hypothesis, transient expression analyses have been initiated to examine the functional contribution of cell type-dependent interactions which occur between cellular factors and the HTLV-I LTR in the regulation of viral gene expression in cells of immune and nervous system origin.

NZ 120 DELETIONAL ANALYSIS OF EXON 2 OF TAT-2 IN TRANS-ACTIVATION OF HIV-1 AND HIV-2 LONG TERMINAL REPEATS. Sandra E. Tong, Barbara Porton, and Jun *Pagtakhan*. UCSF, Dept. of Med., SF VAMC, San Francisco, CA 94121.

High level expression of HIV-1 requires a viral protein, Tat (trans-activator of transcription)-1, which acts on the trans-acting responsive region (TAR)-1 transcribed from positions +1 to +60 in HIV-1LTR. TAR-1 forms a single stem-loop secondary RNA structure. HIV-2 is a second human retrovirus associated with prolonged latent infection. High level expression of HIV-2 also requires its trans-activator, Tat-2; however, TAR-2 transcribed from the HIV-2LTR forms a double stem-loop RNA structure. We studied the function of Tat-2 in trans-activation of HIV-1 and HIV-2 in transient transfection assays in a human CD4+ T cell line, Jurkat. We have found that the second exon of Tat-2 is important for its trans-activation function. Studies with HIV-2LTR show that exon 1 of Tat-2 trans-activates this LTR by 80-fold. The genomic form of *tat*-2 results in comparable trans-activation (116-fold) and full-length Tat-2 trans-activates HIV-2LTR most efficiently (430-fold trans-activation). Similar results are observed with trans-activation of HIV-1 LTR by Tat-2. The intermediate phenotype of Tat-2 expressed from genomic DNA results from incomplete splicing mRNA. Mutagenesis studies show that caboxy-terminal deletion of Tat-2 to residue 119 results in a slight decrease in trans-activation while deletion to residue 110 results in 240-fold trans-activation of HIV-2LTR, or 55% of wild-type Tat-2. Residues 110-119 contain basic residues in exon 2 of Tat-2 which may enhance the binding of full-length Tat-2 to target TAR structures. Thus, the 31 residues encoded by exon 2, including a basic domain, are necessary for optimal trans-activation of HIV-1 and HIV-2 LTRs and obviate the requirement for a double stem-loop target. Differential expression of exon 1 of Tat-2 and full-length Tat-2 may contribute to prolonged latency in the HIV-2 viral life cycle.

NZ 121 **p53 INHIBITS BASAL AND TAX-MEDIATED EXPRESSION OF THE HTLV-1 LTR IN T CELLS**, William Wachsman[1,2], Ping Shen[2], Bryan Strauss[3] and Martin Haas[3], [1]Research Service, San Diego VAMC, [2]Division of Hematology-Oncology and [3]Cancer Center, UCSD School of Medicine, La Jolla, CA 92093-0677.

The human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL), a highly aggressive T-cell malignancy. The HTLV *tax* accessory gene product has been implicated as serving a role in HTLV-induced T-cell transformation and leukemia/lymphoma. Tax appears to function indirectly, through host cell factors, to dysregulate expression of genes involved in T-cell growth and proliferation. The p53 gene product has been shown to inhibit cell growth and proliferation. Quiescent T cells do not express p53, however, following lectin stimulation or infection by HTLV-1 in vitro, expression of wild-type p53 is induced. Several studies have shown that leukemic T cells from ATLL patients often express abnormal p53 protein, suggesting that its mutation may be involved in the development of this HTLV-1-induced malignancy. It is also known that HTLV-transformed T cells expressing wild-type p53 have high levels of Tax expression, while those expressing low levels of wild-type p53 or any amount of mutant p53 have low levels of Tax.

Based on these observations, we sought to determine whether p53 effects the expression of the HTLV-1 LTR in either the presence or absence of Tax in a T-cell system. The Jurkat T-cell line does not express wild-type or mutant p53. Cotransfection of an HTLV-1 LTR/CAT recombinant and an CMV-driven p53 expression construct into this T cell line revealed 5-fold diminution in basal expression of the LTR. In contrast, CMV-Tax1 and CMV-Tax2 induced 8- and 35-fold expression of the LTR, respectively. The addition of CMV-p53 reduced Tax-mediated transactivation of the HTLV-1 LTR in a dose-dependent fashion. By comparison, four different mutant p53 constructs, each expressing an abnormal p53 containing a single amino acid substitution located between p53 residues 175 and 248, failed to reduce basal expression of the HTLV-1 LTR or its transactivation by Tax. Each of these p53 mutants has been reported in malignant cells derived from patients. These data indicate that wild-type p53 inhibits both basal and Tax-induced expression of the HTLV-1 LTR. Several mechanisms could account for this action, the most likely being competition by p53 for a host cell factor or a cis-acting element in the LTR, necessary for its transcription, through which Tax operates. These results support the hypothesis that critical mutations in p53 may play a role in the pathogenesis of HTLV-induced ATLL.

NZ 122 NOVEL SMALL TRANSCRIPTS OF THE HEPATITIS B VIRUS X GENE, T.S. Benedict Yen & Y.W. Zheng, Department of Pathology 113B, VA Medical Center, University of California, San Francisco, CA 94121.

The hepatitis B virus X gene product has been shown to be a transcriptional trans-activator. Recently, Kwee et al. (J. Virol., 66:4382) showed that there are at least 2 forms of the X protein, both of which are necessary for full trans-activation function. One form (here called large X protein) is translated from the first ATG codon, while the other form (here called small X protein) is translated from the third in-frame ATG codon of the X oepn-reading frame. In addition, a third form (here called the middle X protein) may be translated from the second in-frame ATG codon. They had proposed that the middle and small X proteins were synthesized by scanning ribosomes leaking through the first and second ATG codons of the X transcript, respectively. We will present data, showing that there are novel primary transcripts of the X gene, that can be directly translated into the middle and/or small X proteins. Specifically, primer extension analysis revealed transcripts initiating within the X open-reading frame at sites spanning the second ATG codon. These transcripts were not degradation products of larger transcripts, since their synthesis did not depend on up-stream sequences containing the previously mapped X promoter. They were translated to generate protein product(s), as revealed by positive immunofluorescence staining of transfected cells with a monoclonal antibody against the large X protein. Therefore, there appear to be X gene transcripts specifically designed to give rise to middle and small X proteins.

Cellular Targets of DNA Tumor Viruses and the Papillomaviruses

NZ 200 MUTATION OF A CRITICAL GLUTAMIC ACID RESIDUE IN THE 16KDa PORE FORMING COMPONENT OF THE VACUOLAR H+-ATPase REDUCES ITS INTERACTION WITH THE BOVINE PAPILLOMA-VIRUS E5 PROTEIN AND CONVERTS IT INTO A TRANSFORMING PROTEIN.

Thorkell Andresson, Jason Sparkowski, David Goldstein and Richard Schlegel. Department of Pathology, Georgetown University Medical School, Washington DC.

16K is the pore-forming constituent of the vacuolar ATPase. This enzyme complex is responsible for acidifying many intracellular compartments including coated vesicles, endosomes, lysosomes, and the trans-Golgi apparatus. Previously we have shown that the E5 oncoprotein of bovine papilloma virus type-1 (BPV-1) binds to 16K and that the glutamine residue (amino acid #17) in the E5 transmembrane domain is important for this interaction. To further study the specificity of this interaction and to map the E5 binding site on 16K, two mutants were made in the well-conserved fourth transmembrane domain of 16K. One point mutation was made which changed Glu #143 to Arg. In addition, a 16K protein lacking the fourth transmembrane was also made (termination at Ile #136). Co-immunoprecipitation experiments in Cos cells showed that the binding of Arg#143 and truncated 16K was decreased to 30% and 10% respectively, compared to wild type. This strongly suggested that helix four is the binding site on 16K for E5. Based on these results it was suggested that Glu #143 in 16K and Gln #17 in E5 might be contributing to this binding via charge interactions. To evaluate this possibilitly, co-immunoprecipitation studies were done using 16K Arg#143 and two E5 mutants (#17 Glu and and #17Asp). Interestingly, our results show that the binding of these two E5 mutants to the mutated 16K protein was dramatically increased (to 140% of wt 16K levels), suggesting that charge interactions did indeed modulate 16K/E5 binding.

To evaluate whether the generated 16K mutants might interfere with normal 16K function or mimic the activity of E5, we expressed these mutant proteins stably in NIH3T3 cells. Expression of the proteins was verified by immunoprecipitation and the cells were then assayed for growth in soft agar. Our results demonstrate that both the Arg#143 and truncated 16K proteins were able to transform cells. In contrast, NIH3T3 cells stably transfected with either the wild type 16K or vector DNA alone did not. Our findings demonstrate that abnormal 16K function is capable of inducing cellular transfomation and that E5 may function to perturb the activity of this critical element of the vacuolar ATPase.

NZ 201 EPITHELIAL-CELL SPECIFIC ENHANCER ACTIVATION OF HUMAN PAPILLOMAVIRUS-16

Doris Apt, Mark O'Connor, Yichun Liu and Hans-Ulrich Bernard, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511.

Transcription of the Human Papillomavirus-16 (HPV-16) early oncogenes E6 and E7 is regulated by an epithelial specific enhancer. Enhancer activation is dependent on the cooperative interaction of a specific subset of cellular transcription factors present in epithelial cells. Nuclear factor I (NFI) which binds to 7 'TTGGC' motifs in the HPV-16 enhancer shows cell type specific binding profiles in gel retardation assays which correlate with gain or loss of enhancer function. The proline rich activation domains of different splice forms of NFI-C (epithelial type) and NFI-X (fibroblast type) are studied in detail and functional data are presented. However, for optimal enhancer activation, additional binding sites for API, GRE, TEF-1, TEF-2 and Oct-1 are essential. The highly conserved nature of the Oct-1 binding site, positioned exactly 2 bp from the promoter proximal NFI site, suggests an important functional role. Binding profile studies and functional data demonstrate that Oct-1 contributes to HPV-16 enhancer activation indirectly by stabilizing the binding of NFI to its low-affinity, half-palindromic binding sites. The involvement of Oct-1 in cellular and viral proliferation events provides a potential role for this Oct-1/NFI composite element in cell-cycle responsive or differentiation dependent viral transcription.

NZ 202 ONCOPROTEINS OF DNA TUMOR VIRUSES SHARE THE ABILITY TO ELIMINATE THE CYCLIN D1 CHECKPOINT IN G1, Jiri Bartek, Jiri Lukas, Jirina Bartkova, Heiko Müller and Michael Strauss, Division for Cancer Biology, Danish Cancer Society, DK-2100 Copenhagen, Denmark.

The multifunctional oncoproteins of the papilloma, papova and adeno DNA tumor viruses share several features aimed at deregulation of the host cell proliferation control, commonly exerted via disruption of the cell's key regulatory protein-protein interactions which involve the p53 and retinoblastoma tumor suppressors, E2F transcription factor, p107, cyclin A, cyclin E and cdk2 kinase. Now we report that SV40 large T antigen, adenovirus E1A and papillomavirus E7/E6 oncogenes share the ability to downregulate expression of cyclin D1, a key G1 cyclin, in mammalian cells. Functional protein knockout experiments indicate that cyclin D1 plays an essential role in G1 phase progression of both normal cells and cell lines established from several types of human tumors, including those with cyclin D1 gene amplification. This cyclin D1-dependent cell cycle regulatory function appears to be eliminated by E1A, E7/E6 and SV40 T antigen. Our data suggest that the mechanisms utilized by the viral oncoproteins to target cyclin D1 are significantly different from those subverting the functions of cyclins A and E. We propose that the capacity of these viral oncoproteins to downregulate cyclin D1 and bypass the cell's requirement for cyclin D1 function in G1 progression represents a novel molecular mechanism commonly explored by the small DNA tumor viruses as part of their evolutionary successful strategy to subvert the host cell cycle control and facilitate viral replication. Analysis of deletion mutants and gene transfer experiments with cell lines carrying aberrant retinoblastoma gene suggest that the above effects of the viral oncogenes on cyclin D1 abundance and function are mediated via their complex formation with pRB and implicate pRB as a major target of cyclin D1's cell cycle regulation function in G1.

NZ 203 SEQUENCE DIVERSITY IN PAPILLOMAVIRUSES,

Hans-Ulrich Bernard, Lisa Ho, Chi-Keong Ong and Shih-Yen Chan, Institute of Molecular and Cell Biology, National University of Singapore.

The study of molecular diversity between papilloma-virus (PV) types and of independent isolates of each PV type has become a powerful tool to explore the rational behind PV taxonomy, pathways of PV evolution and novel approaches in epidemiological research. Sequence comparison of PVs from different mammalian species apparently points to a strict coevolution of virus and host, suggesting that the peculiarities of PV genome organization are at least 100 million years old. On a more recent level, diversity between human PV types seems to have arisen before evolution of Homo sapiens, and diversity within a type strictly in coevolution with the human races. From this observation one has to conclude that that all HPV types should have originated in Africa, a possibility that is presently an objective of our research. The existing molecular diversity of PV genomes permits novel approaches in epidemiological research, which we use to examine concepts such as sexual or vertical transmission, persistence, latency, and clonality of PV lesions. Ongoing efforts concentrate to identify sequence properties of PV types and of variants of each PV type that correlate with variabilities in pathological properties.

NZ 204 TRANSCRIPTIONAL REGULATION OF HUMAN PAPILLOMAVIRUS (HPV) ONCOGENE EXPRESSION: COMPOSITION OF THE HPV18 UPSTREAM REGULATORY REGION, Karin Butz and Felix Hoppe-Seyler, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, INF 242, D-69120 Heidelberg, Germany.

The transforming activity of human papillomaviruses is closely linked to the expression of the viral E6 and E7 genes. In high risk HPVs, such as HPV16 or HPV18, both genes are transcribed from a common promoter, which is regulated by cis-active elements within the viral transcriptional control region (upstream regulatory region, URR). A number of host cell factors have been implicated to bind to the HPV18 URR, including AP1, Sp1, KRF-1, NF1, Oct-1 and the glucocorticoid receptor. In the present study, the functional significance of these factors for the regulation of E6/E7-promoter was assessed by a systematic mutational analysis of the respective recognition motifs within the physiological context of the complete HPV18 URR. Using transient luciferase reporter assays, the activity of the viral oncogene promoter was found to be largely dependent on the integrity of AP1-, Sp1- and, in some epithelial cells, KRF-1-binding sites. A promoter proximal glucocorticoid response element was indispensable for the hormone responsiveness of E6/E7-promoter. Functional dissection of the HPV18 URR indicates that a thus far undetected cis-active element within the enhancer region plays an important role for the generation of enhancer activity. Finally, comparative analyses employing homologous and heterologous promoters indicate that the HPV18 enhancer is influenced by the nature of the test promoter in a cell type dependent manner.

NZ 205 IDENTIFICATION OF CELLULAR PROTEINS THAT INTERACT WITH THE HPV18 E7 PROTEIN, Kevin G. Coleman, Barri S. Wautlet, David V. Morrissey, Janet G. Mulheron, Sandy Price, Kevin R. Webster and Sylvia A. Sedman. Department of Molecular Genetics and Cell Biology, Bristol-Myers Squibb-Pharmaceutical Research Institute, Princeton, N. J. 08543

An IMR90 (normal human lung fibroblast cell line) cDNA expression library in λgt11 was probed with a bacterially expressed HPV18 E7 fusion protein to identify cellular proteins that interact with E7. Twenty-two independent cDNA clones were isolated. One of these clones encodes the previously identified son3 gene, whose protein product contains limited sequence similarity with Rb and p107. We have shown that a son3 fusion protein can specifically precipitate HPV16 E7 from CaSki cell lysates. Northern analysis indicates that son3 cDNA hybridizes to at least 5 mRNAs, ranging in size from 2.4 Kb to 12.0 Kb, and is expressed in a cell cycle dependent manner. Western blot analysis detects an 86 kDa protein in CaSki and C33a cells but not in HeLa cells. The 86 kDa cellular son3 protein, immunoprecipitated from CaSki cells, binds to the HPV18 E7 protein *in vitro*. In addition, we demonstrate *in vitro* binding between son3 and the Adenovirus E1A protein and show that son3 binds to the nonconserved amino terminal end of AdE1A. This region of AdE1A has been shown to encode transforming activity and is the region to which the p300 protein binds.

NZ 206 A ROLE FOR E2F-1 IN CELL CYCLE PROGRESSION, James V. DeGregori, James K. Schwarz and Joseph R. Nevins, Howard Hughes Medical Institute, Section of Genetics, Duke University Medical Center, Durham, NC 27710

The E2F transcription factor has been identified as an important participant in the control of cellular proliferation. E2F binding sites are found in the promoters of several cellular genes essential for DNA synthesis (e.g. *c-myc*), and these sites are required for the serum stimulation of the DHFR gene. Also, the retinoblastoma protein and the related p107 protein associate with E2F and inhibit E2F transcriptional activity. The products of several DNA tumor viruses disrupt these complexes dependent on regions of the proteins required for their oncogenic potential. Recently, the *E2F-1* gene product, one of the components of the cellular E2F activity, has been shown to induce S-phase in quiescent fibroblasts. Nevertheless, given the fact that E2F-1 is but one of several E2F components, we have sought to determine the requirement for E2F-1 in S-phase induction. Our approach has been to employ E2F-1 antisense oligonucleotides to interfere with E2F-1 protein accumulation. Phosphorothioate C-5 propyne oligonucleotides antisense to *c-myc* or *E2F-1* transcripts microinjected into quiescent human foreskin fibroblasts inhibited the induction of DNA synthesis by serum. Antisense E2F-1, but not antisense c-myc, also inhibited the induction of DNA synthesis by the adenovirus $E1A_{12S}$ product. Similarly, the $E1A_{12S}$ product rescues the inhibition of serum induced DNA synthesis by c-myc antisense, but not E2F-1 antisense. These results indicate that adenovirus circumvents *c-myc* in the induction of DNA synthesis and demonstrates that *E2F-1* is required for the induction of DNA synthesis by both serum and adenovirus in human foreskin fibroblasts. This approach should help establish these and other potential players in pathway(s) for the induction of DNA synthesis.

NZ 207 PAPILLOMAVIRUS DNA IN CHOLESTEATOMA OF THE EAR. Ethel-Michele de Villiers and Krister Bergmann, Division of Tumourvirus-Characterization, Deutsches Krebsforschungs-zentrum, Heidelberg, Germany.

Histologically, papillomatous growth and clusters of koilocytes are the typical features of the aggressively growing, bone-destructive areas of a cholesteatoma of the ear, thus resembling the features cf a papilloma. Biopsies from such tumors were examined by PCR for the presence of papillomavirus DNA. Utilizing degenerate primers, two different HPV types could be detected. The majority of the tumors harbored HPV 11 DNA whereas an yet unidentified papillomavirus was isolated from one case. These data suggest a papillomaviral etiology in the development of cholesteatoma.

NZ 208 THE TRANS-ACTIVATION AND DNA BINDING DOMAINS OF E2 ARE REQUIRED FOR GROWTH SUPPRESSION OF HPV POSITIVE CELL LINES, Jennifer Dowhanick, Alison McBride* and Peter M. Howley, Department of Pathology, Harvard Medical School, Boston, MA 02115, *Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda. MD 20892.

The progression of HPV infected cells to cancer is often associated with the integration of the viral genome resulting in the disruption of the E2 open reading frame. The E2 protein regulates the expression of the viral oncoproteins, E6 and E7. Expression of bovine papillomavirus type-1 (BPV-1) E2-TA in HeLa cells, an HPV-18 cervical carcinoma cell line, results in growth suppression (Thierry, F. and M. Yaniv, 1987, Hwang, E.-S., et al, 1993) and the transcriptional repression of E6 and E7 (Hwang, E.-S., et al, 1993).

We have mapped the domains of BPV-1 E2 which are necessary for growth suppression by co-transfecting HeLa cells with E2 expression vectors and a selectable marker. Although the BPV-1 E2-TA protein has been demonstrated to inhibit growth, the E2 repressor (E2-TR), which inhibits the expression of E6 and E7 does not affect cell growth. Chimeric proteins containing the E2 DNA binding domain and the trans-activation domain from either VP16 or spi also fail to inhibit growth, indicating that the E2 trans-activation domain is necessary for this effect. BPV-1 E2-TA mutants have been used to examine the domains necessary for E2 growth suppression. Finally, the E2 proteins of HPV-16 and HPV-18 have the same growth suppressive effect as the BPV-1 E2-TA and E2 growth suppression can be manifested in other HPV positive cell lines.

NZ 209 THE ROLE OF THE E1 AND E2 GENE PRODUCTS IN THE REPLICATION OF HUMAN PAPILLOMAVIRUS TYPE 31B Mark G. Frattini and Laimonis A. Laimins, Departments of Biochemistry and Molecular Biology and Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637

The roles of the HPV-31b E1 and E2 gene products in the viral DNA replication process have been examined biochemically by overexpressing the two proteins in both a glutathione-S-transferase (GST) fusion-protein system and an SV-40 based eukaryotic expression system. In addition to the full length E1 protein (629 aa), a truncated E1 protein consisting of the first 268 aa (E1*) was synthesized. We show that both the full-length E1 protein and E1* complex efficiently with the E2 protein when transiently expressed in Cos 7 cells and in vitro using purified recombinant protein with in vitro translated protein. Site-specific DNA binding by both the E2 and full-length E1 proteins to a 291 bp fragment containing the sequence analagous to the viral origin of replication for BPV-1 was observed. The E1* protein, however, failed to bind specifically to this DNA fragment. Modified McKay DNA binding experiments demonstrate that the E2 protein stimulates the binding of E1 to DNA. This stimulation is independent of whether E2 is bound to the DNA or complexed with E1 prior to binding DNA. DNase I footprinting of E1 alone versus the E1:E2 complex to origin containing DNA shows that the addition of E2 to the complex alters the pattern of E1 binding to DNA. HPV replication has also been examined using transient assays in a squamous cell carcinoma cell line (SCC-13) as well as primary keratinocytes. In these studies we have demonstrated that expression of both the E2 and full-length E1 proteins are necessary and sufficient for episomal replication of a plasmid construct which includes the 291 bp origin-containing fragment.

NZ 210 MODULATION OF mRNA-TRANSPORT IN ADENOVIRUS-INFECTED CELLS, Stefan Gabler, Thomas Dobner, Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany.

The control of messenger RNA transport from the site of transcription and processing in the nucleus to its ultimate location in the cytoplasm is poorly understood. However, studies on RNA metabolism during adenovirus infection revealed that the virus is capable of specifically modulation both cellular and viral mRNA transport during the course of an infection (host shut-off). In order to elucidate the molecular mechanisms involved we have focused on the adenoviral gene products that mediate this control of RNA transport. Our results suggest a model that explains the ability of the viral proteins to simultaneously facilitate the transport of viral mRNAs and inhibit the transport of cellular mRNAs. We propose that a specific complex of the early viral proteins E1B-55kD and E4-34kD, localized to the sites of viral transcription and processing, usurps cellular components required for the transport of messenger RNA molecules. By directing this factor(s) to the sites of viral RNA transcription and processing, it is no longer accessible to the newly synthesized cellular mRNAs.

Based on these observations we have started to utilize the interactions of the viral gene products with each other and with the host cell as a means of indentifying cellular components that specifically associate with the E1B-E4 complex and participate in the transport of messenger RNA from the nucleus.

NZ 211 THE BPV-1 E5 ONCOPROTEIN COOPERATES WITH THE ß-TYPE, BUT NOT THE α-TYPE, PDGF RECEPTOR TO INDUCE TRANSFORMATION OF A MURINE MYELOID CELL LINE. David J. Goldstein[1], Ling-Mei Wang[2], Weiqun Li[2], Stuart Aaronson,[2] Richard Schlegel,[1] and Jacalyn Pierce[2]. [1]Departments of OB/GYN and Pathology, Georgetown University Medical School, Washington, D.C., 20007. [2]Laboratory of Cellular and Molecular Biology, NCI, NIH, Bethesda, M.D., 20892.

The 44-amino-acid E5 protein of bovine papillomavirus type 1 (BPV-1) is a highly hydrophobic protein that transforms cells through the activation of growth factor receptors. To investigate the ability of the E5 protein to induce mitogenic signalling through growth factor receptors, we utilized a nontumorigenic, murine myeloid cell line (32D) which is strictly dependent on interleukin 3 (IL-3) for sustained proliferation in culture. This dependence can be functionally substituted by the stimulation of signal transduction pathways mediated by a variety of growth factor receptors following addition of the corresponding ligand. Several receptor cDNAs were used to transfect 32D cells constitutively expressing the E5 protein to test for IL-3-independent growth. Of several receptors tested (αPDGF-R, ßPDGF-R, EGF-R, CSF-1-R, and IL-2-R), only the ß-type PDGF receptor was capable of abrogating the dependence on IL-3 for survival in culture. The mitogenic signalling induced by the coexpression of the ßPDGF receptor and E5 was characterized by stable complex formation between these proteins, the specific stimulation of tyrosine phosphorylation of the receptor, and tumorigenicity in nude mice. Surprisingly, the highly related α-type PDGF-R failed to cooperate with the E5 protein to induce IL-3-independent growth. Though E5/αPDGF-R-expressing cells expressed levels of protein comparable to the E5/ßPDGF-R-expressing cells, no E5/αPDGF-R complex formation was detected. These results demonstrate that the E5-induced mitogenic activity in 32D cells is a direct consequence of specific complex formation with ßPDGF-R and the stimulation of its tyrosine phosphorylation.

NZ 212 TRANSCRIPTIONAL TRANSACTIVATION FUNCTION OF ENDOGENOUS P53 IN CERVICAL CARCINOMA CELLS, Felix Hoppe-Seyler and Karin Butz, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, INF 242, D-69120 Heidelberg, Germany.

By employing gel retardation analyses and transient reporter assays, DNA-binding competent and transcriptionally active p53 protein could be detected in HeLa cervical carcinoma cells. The endogenous transactivation function of p53 could be repressed by overexpressing mutant p53 protein, human mdm-2 oncoprotein or HPV16 E6 protein, but not by HPV6 E6 protein. These findings indicate that, in analogy to cellular oncoproteins, the E6 protein of high risk HPVs is able to interfere with the endogenous p53 function in cervical carcinoma cells. The finding that HeLa cells, despite the presence of high risk HPV18 E6 sequences, still contain transcriptionally active p53, raises the question whether E6 at physiological levels is able to interfere with the transactivation function of endogenous p53. Therefore, the endogenous p53 function was assessed in a series of additional HPV positive or HPV negative cervical carcinoma cells and compared with non-tumorigenic cells containing wildtype p53 alleles. These experiments indicate that tumorigenicity does not necessarily correlate with the basal level of p53 transactivation function. Studies investigating the transactivation properties of endogenous p53 after treatment of these cells with DNA-damaging agents will be presented.

NZ 213 CHARACTERIZATION OF E6-AP, AN E3 UBIQUITIN PROTEIN LIGASE THAT MEDIATES THE HPV E6-DEPENDENT DEGRADATION OF P53. Jon Huibregtse, Martin Scheffner*, and Peter Howley. Department of Pathology, Harvard University Medical School, Boston MA 02115, and *Deutsches Krebsforschungzentrum, D-6900 Heidelberg 1, Germany.

The human papillomavirus (HPV) types 16 and 18 are associated with cancers of the ano-genital tract, including cervical cancer. The E6 and E7 proteins encoded by these viruses have been shown to contribute to the immortalization of squamous epithelial cells, the normal host cell for the virus. The E6 proteins are thought to function in immortalization, at least in part, by complexing with and inactivating the p53 tumor suppressor. A 100 kd cellular protein, E6-AP, is involved in mediating the association of E6 with p53. p53 is specifically targeted for ubiquitination following formation of an E6/E6-AP/p53 ternary complex, which leads to the subsequent proteolysis of p53.

Ubiquitination reactions are catalyzed by a class of proteins called E2 ubiquitin conjugating enzymes and often require an additional E3 activity, which allows an E2 protein to specifically recognize the substrate. E6 and E6-AP can stably interact with each other and we have shown that this complex has E3 activity with respect to the ubiquitination of p53. The specific E2 enzyme that is involved is a previously undefined human E2 similar to Arabidopsis UBC8. Additionally, E6-AP can stimulate the ubiquitination of proteins even in the absence of E6, suggesting that the normal function of E6-AP is that of an E3.

Functional analysis of E6-AP has defined domains responsible for E6 binding, p53 binding, and ubiquitination activity. Database searches have revealed several proteins of unknown function which are similar to E6-AP over the region that is important for ubiquitination activity. E6-AP and these E6-AP-related proteins may therefore represent a class of proteins that function in targeting cellular proteins for ubiquitin-mediated degradation.

NZ 214 **G1-specific cyclin D1 activates E2F-dependent transcription on DHFR- and E2-promoters and can bind in vitro to E2F-1**

P. Jansen-Dürr, A. Schulze, D. Spitkovsky, K. Zerfaß (Deutsches Krebsforschungszentrum, Heidelberg, Germany)

One of the targets of transforming DNA-viruses is the transcrption factor E2F. As it was shown before, E2F-activity is cell-cycle regulated and cotrols the expression of several genes involved in celluar proliferation (c-myc, N-myc, DHFR etc.). During different phases of the cell-cycle E2F can be found in association with other proteins that are involved in growth control: pRb, cyclin A, cyclin E, cdk2 and the pRb-related protein p107.

In transient cotransfection experiments in rodent fibroblasts (NIH 3T3 cells) we analysed the activity of the E2F-dependent promoters E2 and DHFR in response to expression plasmids coding for proteins that might regulate E2F. Cotransfection of cyclin D1 but not cyclin A leads to a strong increase in expression of reporter genes driven by E2 and DHFR promoters (20 and 8 fold, respectively). This activation did not occur in pRb-deficient Saos2- or C33A-cells, which suggests that pRb is involved in the transcriptional response.

In gel-shift experiments with extracts from primary human fibroblasts and NIH 3T3-cells, using the E2F-binding site from the E2 promoter as a probe, we were able to show that one of the complexes specific for E2F is recognized by antibodies raised against the C-terminus of cyclin D1. Binding studies with in vitro translated cyclin D1 and bacterially produced GST-E2F1 fusion protein revealed that cyclin D1 can bind directly to E2F1 and suggests that this interaction leads to the activation of E2F-dependent transcription.

NZ 215 INTEGRATION OF HPV-16 DNA INTO THE HUMAN GENOME LEADS TO INCREASED STABILITY OF E6/E7 mRNAS: IMPLICATIONS IN CERVICAL CARCINOGENESIS, Saewha Jeon and Paul F. Lambert, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison,WI 53706

In many cervical cancers, HPV-16 DNA is found to be integrated into the host chromosome in contrast to the extrachromosomal state of HPV DNA in productively infected tissue. This change in genomic state has been argued to result in the altered expression of the viral oncogenes, E6 and E7. We demonstrate here that this altered expression may result in part from the increased stability of E6/E7 mRNAs. We derived clonal cell populations of HPV-16 infected human cervical epithelial (W12) cells which harbor multiple copies of either predominantly extrachromosomal or purely integrated HPV16 DNA. Similar to what is seen in HPV-16 $^+$ cervical cancers, two different patterns of HPV16 DNA integration were observed among the purely integrated clonal populations: either a) all copies of HPV-16 DNA were disrupted by integration in the E1 and/or E2 translational open reading frames, or b) multiple, tandemly arrayed, unit-length copies of HPV-16 genome were found positioned at a site flanked by a disrupted copy of the HPV-16 genome. Surprisingly, the E6/E7 specific mRNAs present in both clonal populations (a or b) arose predominantly from the disrupted, junction copy of the HPV-16 genome, based upon Northern and S1 analyses. Measuring mRNA half-lives using actinomycin D, we found E6/E7 mRNAs present in the integrated clonal cell populations to be 2-6 fold more stable than the E6/E7 mRNAs present in the clonal cell populations harboring intact extrachromosomal viral DNA. We propose that the increased stability of the E6/E7 mRNA arising from the disrupted viral genome results from the loss of a mRNA instability element located in the 3'UTR of HPV-16 early mRNAs.

NZ 216 HUMAN PAPILLOMAVIRUS TYPE 16 DNA CAN IMMORTALISE HUMAN ENDOTHELIAL CELLS *IN VITRO*.

Richard J Jewers[1], Neville A Punchard[2], Barbara Kell[1], Duncan J Watson[2], Matthew D Sankey[2], Richard PH Thompson[2] & Jennifer M Best[1]. The Richard Dimbleby Laboratory of Cancer Virology[1] and Gastrointestinal Laboratory[2], The Rayne Institute, UMDS, St Thomas' Hospital, London SE1 7EH, UK.

Vascular endothelial cells are critical in the control of blood flow, leucocyte recruitment into inflamed sites, thrombosis, coagulation, angiogenesis and hence important in inflammation, wound healing and cancer. There is thus much interest in modelling their behaviour *in vitro*. Human umbilical vein endothelial cells (HUVECs) are commonly used for such purposes but their isolation is time consuming, requires access to human tissue and there are large differences in behaviour of cells isolated from different individuals. Immortalised endothelial cells would greatly facilitate these studies.

The ability of human papillomavirus type 16 (HPV-16) DNA to immortalise epithelial cells is well documented but the effect of this viral DNA in endothelial cells is hitherto unknown. We have demonstrated that HUVECs transfected with HPV-16 DNA have a greatly increased lifetime in culture. Furthermore, the transfected cells retain endothelial cell characteristics: they bind the lectin *Ulex europaeus* agglutinin I, stain positive for von Willebrand factor and possess Weibel-palade bodies. Thus HPV-16 transfection provides endothelial cells with an extended lifetime that may be useful for the *in vitro* investigation of vascular biology.

Results will be presented as to which of the three HPV-16 oncoproteins, E7, E6 and E5, are sufficient for the immortalisation of HUVECs.

NZ 217 THE ADENOVIRUS E2a PROMOTER AS A PROBE FOR THE STUDY OF F9 CELL DIFFERENTIATION, Claude Kedinger, José L. Bocco, Bruno Chatton, Bernard Reimund, Jean Goetz and Rahul Gopalkrishnan, LGME (CNRS), U. 184 (INSERM), 11 rue Humann, 67000 Strasbourg, France

Transcriptional activation of the adenovirus E2a promoter by E1a correlates, on one hand, with the ability of both early E1a proteins (289R and 243R) to sequester the retinoblastoma susceptibility gene product (Rb) and thereby release E2F from inactive complexes with this protein. On the other hand, only 289R stimulates this promoter by actively interacting with the ATFa proteins, members of the ATF/CREB family. In addition to its responsiveness to the E1a proteins, the E2a promoter is efficiently stimulated by a product of the viral E4 transcription unit, a 17.5 kD polypeptide encoded by the open reading frame 6/7 (E4-ORF6/7). The E4-dependent activation results from specific interactions between the E2F and E4-ORF6/7 proteins leading to the formation of complexes which bind cooperatively and stably to the two neighbouring E2F binding sites in the E2a promoter.

We have previously shown that in **undifferentiated F9 cells** (F9EC), the E2a promoter is refractory to E1a, but not to E4-ORF6/7, responsiveness to both inducers being recovered only after differenciation of these cells.

(i) We show that the ATF-dependent activation of E1a is restored in F9EC cells, in the presence of okadaic acid, an inhibitor of protein-phophatases. By contrast, treatment of differentiated cells with staurosporine, an inhibitor of protein-kinases, abolishes E1a-responsiveness. The role of a protein-kinase activity strongly associated with ATFa, in the mechanism of the cell differentiation-dependent action of E1a, is being examined.

(ii) Using both band-shift and transfection experiments, we show that in F9EC cells, the E4-ORF6/7 product recruites the Rb protein into a stable multi-protein complex with E2F and that in these cells, as opposed to differentiated cells, Rb is actively involved in the transcriptional stimulation of the E2a promoter by E4. Our results suggest that, in undifferentiated cells, the depending on the cell state, Rb may behave either as a transcriptional activator (F9EC cells) or as an inhibitor (differentiated cells).

NZ 218 DETECTION OF E5 PROTEIN IN HUMAN PAPILLOMAVIRUS TYPE 16 (HPV-16) POSITIVE CERVICAL INTRAEPITHELIAL NEOPLASIA (CIN) EXFOLIATED CELLS. B Kell, R J Jewers, J Cason, F Pakarian and J M Best. The Richard Dimbleby Laboratory of Cancer Virology, The Rayne Institute, St Thomas' Hospital, UMDS, London, SE1 7EH, UK.

E5, the minor transforming gene of HPV-16 is usually deleted following genome integration and is therefore seen to be unimportant for maintenance of cervical carcinoma. However since the most abundant mRNA transcripts in low grade CIN lesions are E5 and E4[1] we have collected samples of exfoliated cells from patients with a recent history of low grade CIN to examine the possibility of E5 protein expression at this early stage of tumour development. The presence of HPV DNA in the cells was determined using consensus primers (MY09 and MY11) in a PCR and positives further typed using a multiplex PCR as described by Van Den Brule *et al* [2]. E5 protein expression in HPV-16 positive, HPV negative and HPV-18 positive cervical scrapes has been determined in western blot assays using a rabbit polyclonal antisera (PE-6) which was raised to a full length HPV-16 E5 synthetic peptide.

Preliminary results show the presence of a single band at approximately 20kD in 2 of 6 HPV-16 positive patients, suggesting dimerisation of E5 protein *in vivo*. No bands have been detected in HPV-18 positive or HPV negative samples.

[1] Stoler M *et al* (1992) Human Pathology 23 117-127
[2] Van Den Brule A J C *et al* (1990) J Clin Micro 28 2739-2743

NZ 219 TUMORIGENESIS IN TRANSGENIC MICE CARRYING HUMAN PAPILLOMAVIRUS TYPE 16 E6 AND E7 ONCOGENES: INCIDENCE OF RETINOBLASTOMAS AND SQUAMOUS CELL CARCINOMAS.

P. Lambert[1], Jacques Lasudry[3], Dan Albert[3], Mark Jackson[2], Amy Liem[1], Renee Herber[1], and Anne Griep[2]. McArdle Laboratory for Cancer Research[1], Dept of Anatomy[2], and Dept of Ophthalmology[3], University of Wisconsin, Madison, WI 53706 USA

The HPV-16 E6 and E7 genes are thought to be important factors in the development of HPV associated cervical carcinomas. We have generated transgenic mice to assess the *in vivo* activities of these viral oncogenes. Expression of E6 and E7 in different tissues was found to potentiate the development of multiple types of cancers including squamous cell carcinomas and retinoblastomas. The skin cancers arise in mice that earlier in life develop epidermal hyperplasias. Wounding was found to be a co-factor in cancer development. The p53 and retinoblastoma tumor susceptibility genes were not mutated in these cancers consistent with the notion that the trans-dominant activities of E6 and E7 protein function obviate this requirement. The individual activities of HPV-16 E6 and E7 in the skin have been compared to their combinatorial effects. Retinoblastomas were found to develop in one line of transgenic mice with high penetrance. These tumors were found frequently to invade into the brain, and metastasize to lymph nodes. Incidence of retinoblastomas was dependent upon mouse genetic background: the genetic basis for this differential susceptibility will be described.

NZ 220 MUTATIONS AT THE P53 GENE OCCUR AT THE LATEST STAGES OF CERVICAL MALIGNANT PROGRESSION, Stella Mitrani-Rosenbaum and Rimona Tsvieli, The Unit for Development of Molecular Biology and Genetic Engineering, Hadassah University Hospital, Mount-Scopus, Jerusalem, Israel

Somatic mutations of the human gene for p53 are found at high frequency in many human cancers. However, somatic p53 mutation is very rare in cervical carcinomas which carry human papillomavirus, while it can be detected frequently in HPV negative tumors. Primary cervical cancers can develop through a progressive mechanism, from low grade lesions to invasive carcinomas. In order to determine wether p53 somatic mutations can also occur at the first stages of progression towards malignancy, we have analyzed the status of both the p53 and the HPV genes in 25 biopsies or histological sections from cervical tissues at the various stages of malignant progression: 4 normal tissues, 5 benign condylomata acuminata, 4 invasive squamous carcinomas, 6 CIN I/II, 4 CIN II/III and 2 CIN III. The cDNA fragment of the p53 gene was sequenced from codon 237 to codon 390. Only WILD P53 alleles were found in all specimens, including 3 cases of CIN I/II, 3 cases of CIN II/III and 1 case of CIN III, which did not contain HPV sequences. These studies may indicate that the p53 mutations present in most HPV negative primary cervical tumors occur rather late in the progressive malignant pathway.

NZ 221 FUNCTIONAL ANALYSIS OF HPV16 E6 AND E7 ONCOPROTEINS. Nicola Marston, Rachel Davies, Tim Crook, Eric Lam, Jonathan Morris, Roger Watson and Karen Vousden. Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, London W2 1PG, UK.

Human Papillomavirus type 16 (HPV16) encodes two principal oncoproteins, E6 and E7, which are implicated in the development of anogenital carcinoma. Both these viral proteins show immortalising and transforming properties *in vitro* and specifically target tumour suppressor proteins.

HPV16 E6 binds and directs the degradation of p53, abrogating its growth suppressor activity. In order to investigate this interaction, and also that of p53 with its negative regulator mdm-2, a series of p53 mutants targeting the conserved regions of the protein were constructed, and their functional properties assessed. It was shown that E6 and mdm-2 bind independently to p53 *in vitro*, and that although the conserved box deletions which caused p53 to lose ability to bind E6 also lost biological activity, there is not a simple correlation between transformation suppression and transcription activation. The p53 conserved box I deletion mutant which lost mdm-2 binding retained both transcriptional activation and growth suppression acitvity. Mdm-2 is therefore not a downstream effector of p53, and loss of mdm-2 regulation might even enhance p53 growth suppressor activity.

HPV16 E7 associates with regulators of the transcription factor E2F, RB and p107 and we investigated whether these E7 interactions affect the expression of E2F responsive genes. We have shown that the E2F regulated gene B-*myb* is a target for transcriptional activation by E7, leading to both inappropriate transcription of B-*myb* during G1 and constitutive over-expression in cycling cells. This activation correlates with the ability of E7 to interfere with regulatory p107:E2F complexes at the B-*myb* promoter. The proposed role of B-Myb in cell proliferation suggests that activation by E7 is likely to contribute to the mitogenic activity of the viral oncoprotein, and studies have been initiated to correlate this with malignant potential.

NZ 222 CIS-ELEMENTS REQUIRED FOR THE REPLICATION OF HPV-11 ORIGIN. Dennis J. McCance[1], James Lu[1], Yong-Nian Sun[1], Robert C. Rose[2], William Bonnez[2]. Departments of Microbiology and Immunology and Infectious Diseases, University of Rochester, Rochester, NY 14642.

Human papillomaviruses (HPV) cannot be propagated in *in vitro*, but the DNA can be replicated transiently in an assay, in the presence of two *trans* acting viral proteins E1 and E2. Using this assay we have defined the minimal *cis* acting elements of the origin of replication of HPV-11. Most HPV genomes are conserved at the origin of replication and the core contains three E2 binding sites (E2BS) surrounding an A/T rich spacer region. The present results show that the minimal requirement for replication is either two E2BS alone, or the A/T rich region plus one E2BS, in which case the the relative position of the E2BS is important. In all the studies, the presence of both E1 and E2 proteins was essential for replication, yet only E2BS was required at the origin. We have shown that E1, E2 and origin of replication containing an E2BS form a complex *in vitro* and our data are consistent with a model in which E2 acts to target E1 to the HPV-11 replication origin.

NZ 223 ASSOCIATION OF CYCLIN E WITH HUMAN PAPILLOMAVIRUS TYPE 18 E7: MECHANISM OF INTERACTION AND ASSOCIATED KINASE ACTIVITY, Maritza C. McIntyre and Laimonis A. Laimins, Committee on Virology, University of Chicago, Chicago, Illinois, 60637.

The human papillomavirus (HPV) E7 protein has been shown to associate with cyclin A and p33 cdk2. This complex is believed to contribute to the E7 associated histone H1 kinase activity that is maximal at the G2/M phase of the cell cycle. We have identified the G1 cyclin, cyclin E, as another E7 associated protein by western blot analysis of cellular proteins bound to a GST-E7 fusion protein. E7 binds a slow migrating form of cyclin E exclusively. To study the mechanism of this association, we mixed bacterially expressed E7 and cyclin E *in vitro* and found that they do not interact directly. Addition of bacterially expressed p107 to a mixture of E7 and *in vitro*- translated cyclin E allowed the coprecipitation of cyclin E with E7. Additionally, E7 mutants that no longer bind p107 also lose the ability to bind cyclin E. Thus, the association of E7 with cyclin E is not direct, but is mediated by the interaction of both proteins with p107, which can bind either protein independently. E7 also has an associated kinase activity during G1 which is dependent on the E7/cyclin E association. Mutant E7 proteins which no longer bind p107 and cyclin E lose the ability to phosphorylate E7 associated proteins in an *in vitro* kinase assay.

NZ 224 WILD-TYPE p53 STIMULATES EXPRESSION OF GENES INVOLVED IN THE DIFFERENTIATION OF HUMAN EPITHELIAL CELLS, J. A. Mietz and P. M. Howley, Laboratory of Tumor Virus Biology, NCI, Bethesda, MD 20892

Infection by papillomaviruses can alter the normal differentiation pattern of squamous epithelial cells. While this group of viruses generally cause benign proliferative lesions, certain human papillomavirus (HPV) types, such as HPV-16 and 18 are associated with an increased risk for neoplastic progression. The efficient immortalization of epitheial cells by oncogenic HPV types requires the products of two viral transforming genes, E6 and E7. The E6 oncoproteins of HPV-16 and 18 have been shown to form stable complexes with wild-type p53 in vitro and promote the proteolytic degradation of this protein. Wild-type p53 is thought to be an important determinant in the control of cell growth and proliferation. Wild-type p53 has been found to bind to DNA in sequence-specific manner and the results from a number of recent studies indicate that p53 functions as a transcriptional activator. We have recently reported that coexpression of HPV-16 E6 abrogates the transcriptional activation function of p53. For those experiments p53 transactivation was assayed using an artificial p53-responsive reporter gene containing multiple copies of the p53 consensus binding motif. In the present study the ability of p53 to modulate the expression of specific cellular genes involved in the differentiation of squamous epithelial tissues was examined. We have identified at least two cellular genes whose expression is stimulated by wild-type p53. Expression of these two cellular genes is generally limited to the basal cell layer of stratified epithelia where p53 expression is primarily detected. Localization of the p53 target sequence(s) within these genes is currently in progress.

NZ 225 A CELLULAR PROTEIN THAT INTERACTS WITH THE HPV18 E7 PROTEIN CONTAINS A REGION OF HOMOLOGY WITH A PURINE BIOSYNTHETIC ENZYME, David V. Morrissey, Barri S. Wautlet, Janet G. Mulheron, Sandy Price, Kevin R. Webster, Sylvia A. Sedman, and Kevin G. Coleman. Department of Molecular Genetics and Cell Biology, Bristol-Myers Squibb-Pharmaceutical Research Institute, Princeton, N. J. 08543

An IMR90 (normal human lung fibroblast cell line) cDNA expression library in λgt11 was probed with a bacterially expressed HPV18 E7 fusion protein to identify cellular proteins that interact with E7. Twenty-two independent cDNA clones were isolated. Five of these clones represent a single gene encoding a protein (E7BP9) which contains a region of extensive homology with the aminoimidazole ribonucleotide synthetase (AIRS) domain of the human GART protein. The AIRS domain is one of three functionally distinct domains of the GART protein which catalyze separate steps in the *de novo* purine nucleotide biosynthetic pathway. The E7BP9 protein also shares a region of homology with the human Son3 protein, another cellular protein which we have shown to interact with HPV16/18 E7. Northern analysis shows that the E7BP9 cDNA hybridizes to a mRNA of 4.5 Kb, which is expressed in a cell cycle dependent manner.

NZ 226 STRUCTURE-FUNCTION ANALYSIS OF THE CARBOXY TERMINAL DOMAIN OF THE HUMAN PAPILLOMAVIRUS E7 ONCOPROTEIN, Karl Münger[1], Karen E. Clemens[2], Kreton O. Mavromatis* and Carole L. Yee[3]; Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda MD 20892. Present Address: [1]Pathology Department, Harvard Medical School, Boston MA 02115, [2]Laboratory of Molecular Virology, National Cancer Institute Bethesda MD 20892,[3] Laboratory of Dermatology, National Cancer Institute Bethesda MD 20892; *HHMI-NIH Research Scholar.

The transforming functions of E7 are at least in part related to its ability to interact with several host cellular proteins including the retinoblastoma tumor suppressor protein pRB. The binding of HPV E7 to pRB causes the displacement of cellular proteins that are normally complexed to pRB, including the transcription factor E2F-1. Complex formation of E7 with pRB also results in the abrogation of pRB's non-specific DNA binding property. Sequences in the carboxy terminal region of E7, in addition to the pRB binding site in the amino terminus are important for both of these functions. The carboxy terminal sequences may also play a role in the immortalization of primary human genital keratinocytes in cooperation with E6. To study whether specific amino acid sequences are necessary for the functions associated with the carboxy terminus of E7, we replaced this domain with a structurally similar sequence motif derived from the HPV E6 oncoprotein. The resulting E7-E6 chimeras, like the full length E7 protein, can bind pRB *in vitro* and transform baby rat kidney cells in cooperation with *ras*. They were also tested for E7 carboxy terminus-associated functions such as pRB-E2F complex disruption and the abrogation of pRB's non-specific DNA binding property. The results indicate that these two properties are not absolutely required for the transformation function of E7. In collaboration with Roger Brent's laboratory (MGH, Boston) we have used the "interaction trap" method to study the dimerization property of E7. This method involves the generation of two hybrid proteins in *S. cerevisiae* which, when associated, constitute a functional transcriptional activator. Using different domains of the E7 molecule as well as a number of full-length E7 mutants in this system we have shown that dimerization requires the cysteine rich, carboxy terminal domain of E7. This is in agreement with a published biochemical study (McIntyre *et al.*, 1993, J. Virol. **67**., 3142).

NZ 227 MECHANISM OF CERVICAL CARCINOMA CELL GROWTH INHIBITION BY THE PAPILLOMAVIRUS PROTEIN, E2, Lisa Kay Naeger, Eun-Seong Hwang, and Daniel DiMaio, Department of Genetics, Yale University School of Medicine, New Haven, CT 06510

Human papillomaviruses (HPV) have been linked to the majority of cervical cancers, and two HPV oncogenes, E6 and E7, are expressed in most of these carcinomas. Interestingly, the viral gene encoding the E2 regulatory protein is often disrupted by viral DNA integration in the carcinomas, suggesting that loss of E2 function may be an initial step in cancer progression. We have shown that the Bovine papillomavirus (BPV) E2 protein, introduced into HeLa cervical carcinoma cell line via a BPV-SV40 recombinant virus, results in the dramatic inhibition of E6 and E7 expression and of cellular growth. In addition, the proliferation of HT-3 cells, a cervical carcinoma cell line which has no detectable HPV DNA, is also inhibited when BPV E2 is expressed. We are examining which properties of BPV E2 are responsible for this inhibition in cervical carcinoma cell proliferation by screening a panel of E2 mutants which are defective for viral replication and/or transactivation. The effects of these E2 mutants are being analyzed in both HeLa and HT-3 cells to determine the functions of E2 required for it to exert both HPV-dependent and HPV-independent growth inhibitory effects. The E2 proteins from different papillomavirus types are relatively well conserved, so we are also analyzing E2 proteins from HPV type 16,18, and 8, to see if they exhibit a similar growth inhibitory effect as the BPV E2 protein. Examining E2's roles in cellular growth inhibition may provide key information necessary in understanding the switch from normal cell growth to tumor formation.

NZ 228 THE E6-E7 PROMOTER OF EXTRACHROMOSOMAL HPV16 DNA IN CERVICAL CANCERS ESCAPES FROM CELLULAR REPRESSION BY MUTATION OF TARGET SEQUENCES FOR YY1, Herbert Pfister, Michael May, Xiaoping Dong, Frank Stubenrauch, and Pawel G. Fuchs, Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Erlangen, Germany.

Human papillomavirus 16 (HPV16) induces intraepithelial lesions of the cervical mucosa which may develop into invasive cancer. Integration of viral DNA into the host cell genome is believed to be an important step in tumor progression, because it frequently destroys the viral genes E1 and E2, whose products repress the transcription of viral oncogenes. About 30% of HPV16 positive cervical cancers contain only extrachromosomal viral DNA, however, raising questions about alternative possibilities to change the transcription rate. We have recently identified three binding sites for the cellular transcriptional modulator YY1 between nucleotide positions 7779 and 7848 of the HPV16 long control region (LCR) using band-shift experiments with purified YY1 protein and wild-type or mutated oligonucleotides. A LCR fragment comprising these YY1 binding sites was cloned in front of the heterologous tk-gene promoter and suppressed CAT expression three to four-fold. This silencer activity was abolished by a mutation in the second YY1 binding site, which defines an efficient negative control element dependent on the cellular YY1 protein. We amplified the LCRs of episomal HPV16 DNAs from eight cervical cancers by PCR and cloned it into the Bluescribe vector. In four cases sequence analysis revealed deletions or mutations affecting the YY1 binding sites: 1. deletion of nucleotides 7794-7901, 2. two deletions from 7690 to 7805 and from 7788 to 7854 in different molecules, 3. point mutation at position 7796 (binding site 2), 4. point mutation at position 7843 (binding site 3). The expression of reporter genes under the control of mutated LCRs turned out to be 3.5 to 5-fold higher than under the control of HPV16 wild-type LCRs. This suggests that deletion or mutation of target sequences for the cellular repressor YY1 represents a new, repeatedly used strategy of HPV16 to increase its transcription rate.

NZ 229 REGULATION OF JC VIRUS BY THE POU-DOMAIN TRANSCRIPTION FACTOR TST-1

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The human papovavirus JC (JCV) infects 70% to 90% of the population, as shown by the level of seropositivity in adults. Exposure to JCV usually occurs early in life and results in the persistence of an archetypic virus. Reactivation of the latent virus in individuals suffering from immunosuppression can lead to selective infection and cytolytic destruction of the myelin-producing oligodendrocytes of the central nervous system, resulting in the severe demyclinating disease, progressive multifocal leukoencephalopathy (PML). JCV isolated from PML patients exhibits a marked specificity for glial cells, which is mainly defined on the level of transcription. Understanding the pathophysiology of PML might be greatly enhanced by identifying the transcription factors that are involved in determining the glia specificity of JCV. One of the few transcription factors, which is present in oligodendrocytes and at the same time capable of stimulating the promoters of JCV, is Tst-1, also known as SCIP or Oct-6, a member of the POU-domain family. The level of Tst-1 in mature oligodendrocytes, however, is relatively low. To understand how Tst-1 could activate viral gene expression despite its low abundance, we carried out cotransfection experiments with JCV-promoters, Tst-1 and JCT-antigen. At low concentrations of JCT-antigen and Tst-1, as they occur early during infection in oligodendrocytes, synergism between both proteins was observed. Using a series of deletion mutants of both proteins, the regions of Tst-1 and JCT-antigen responsible for the synergism, could be identified by coimmunoprecipitation and GST-affinity chromatography. The importance of the synergistic interaction for the life cycle of JCV will be discussed.

NZ 230 REGULATION OF HUMAN PAPILLOMAVIRUS TYPE 6 GENE EXPRESSION, Ann Roman and Scott Pattison, and Darron Brown, Departments of Microbiology and Immunology and Medicine, Indiana University School of Medicine, Indianapolis, IN 46202.

The replication cycle of human papillomaviruses is tightly linked to the state of differentiation of its host cell, the keratinocyte. There is a low level of viral transcription in undifferentiated basal cells; transcription increases as the cells differentiate with a concomitant increase in viral DNA synthesis. Initial studies of HPV 6 transcription indicated that the 5' half of the long control region (LCR) decreased expression of a reporter gene in transient transfections. In contrast, the 3' half of the LCR contained net enhancer activity. We hypothesize that protein:DNA interactions in this silencer region contribute to the low level gene expression in basal cells. Therefore we have focused our attention on the 5' half of the LCR. By testing a series of 5' and 3' nested deletions, the silencer was narrowed to a stretch of 66 nucleotides within an alternating purine-thymidine rich sequence. When the 5' half of the LCR contained an internal deletion of 94 base pairs including the putative 66 base pair silencer region it did not have silencing activity. This suggests that this region is necessary for silencing activity. In parallel we have used PCR to determine the nucleotide sequence of this region of the LCR in 15 HPV 6-containing biopsies. This analysis indicates that this region of the LCR is conserved (although variations are detected at other sites). We are currently determining whether this region is sufficient for silencing activity. Subsequently we will pursue an analysis of the proteins interacting with this region.

NZ 231 THE E1 AND E2 REGULATORY GENES OF HUMAN PAPILLOMAVIRUS TYPE 16 REPRESS VIRAL IMMORTALIZATION CAPACITY BY SEVERAL MECHANISMS, Helen Romanczuk and Peter M. Howley, Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, MD, 20892

Human papillomavirus types 16 (HPV-16) has been etiologically implicated in the majority of human cervical carcinomas. In these cancers, the viral DNA is often integrated into the host genome in a manner that leads to the disruption of the E1 and E2 genes, suggesting that the disruption of these regulatory genes plays an important role in carcinogenic progression. We have previously shown that the disruption of either the E1 or E2 gene of a wild-type HPV-16 genome markedly increased viral immortalization capacity. One mechanism of repression of immortalization occurs through the binding of E2 to consensus sites adjacent to the major promoter, P_{97}, resulting in the decreased transcription of the transforming genes. The mutation of these E2 binding sites only partially relieved the negative effect of E2 on viral immortalization efficiency, implicating other mechanisms of E2 repression. We have now tested whether E1- and E2-mediated repression of HPV-16 immortalization efficiency acts through additional regulatory sequences in the viral LCR or in a manner independent of the control region. Under the regulation of a strong heterologous promoter, an HPV-16 E1- or E2-mutated genome is only 2- to 3-fold more efficient at immortalization than the wild-type genome, suggesting that the viral transcriptional regulatory region is the major target for E1 and E2 regulation of HPV-16 immortalization capacity. We are now testing whether the control region of another "high risk" papillomavirus, HPV-18, can substitute for that of HPV-16 in E1- or E2-mediated repression of immortalization. The HPV-18 genome is 10- to 50-fold more efficient than HPV-16 for immortalization, and the majority of this difference is due to the viral regulatory region upstream of the transforming genes. A comparison of the modulation by E1 and E2 in each of these constructs may provide evidence for an additional mechanism of E1- and E2- mediated repression of HPV-16 immortalization capacity. Our results provide genetic evidence that a selective growth advantage is provided by integration of the viral genome in a manner that causes loss of expression of either E1 or E2.

NZ 232 HUMAN PAPILLOMAVIRUS E6/E7 ONCOGENES AND THE P53 MEDIATED RESPONSE TO DNA DAMAGE, Robbert J.C. Slebos, Theodore D. Kessis, Lora Hedrick, and Kathleen R. Cho, Department of Pathology, The Johns Hopkins University, 720 Rutland Avenue, Baltimore, MD 21205.

The E6 oncoprotein of high-risk HPV16 is capable of disrupting the p53 mediated cellular response to DNA damage in RKO colorectal carcinoma cells (Kessis *et al.* PNAS (1993) 90:3988-3992). To test whether low-risk HPV11-E6 and high-risk HPV16-E7 could also interfere with this pathway, we expressed these proteins in RKO cells. The resulting transfectants were investigated for the accumulation of *wt*p53 protein and for G_1-cell cycle arrest after sub-lethal DNA damage.

In contrast to 16E6 harboring lines, *wt*p53 protein was easily detectable by ECL-Western blotting in cells expressing 11E6, 16E7 or in control cells transfected with vector alone. After exposing the cells to 4 Gy of γ-irradiation, Western blot analysis revealed that *wt*p53 accumulated in the 11E6, 16E7 and control cells. Prolonged exposure of the ECL-Western blot showed that 16E6 expressing cells had very low levels of *wt*p53 protein, and that these levels increased after sub-lethal DNA damage. Flow cytometric analysis of the transfected lines revealed that the 11E6 and control cell lines arrested in G_1 after 4 Gy of γ-irradiation, whereas the 16E6 lines did not arrest. The 16E7 transfected lines also lost the ability to arrest in G_1, despite increased levels of *wt*p53. All the cell lines arrested in the G_2-phase of the cell cycle, a response that is independent of p53 status.

Our results show that both E6 and E7 from an oncogenic HPV can abolish an important cellular response to DNA damage, whereas E6 from a low-risk HPV type lacks this potential. Presumably cells with disrupted cell cycle regulation are unable to repair damaged DNA prior to replication, allowing fixation of genetic lesions in daughter cells. That infection by HPV may predispose cells to genetic alterations is consistent with the notion of multi-step tumorigenesis, and may be particularly crucial for uterine cervix tumor progression where HPV infection appears to serve as the initiating event in most if not all cases.

NZ 233 MUTAGENESIS OF GLUTAMINE17 IN THE BOVINE PAPILLOMAVIRUS E5 ONCOPROTEIN AND ITS EFFECTS ON TRANSFORMING ACTIVITY AND BINDING TO ITS CELLULAR TARGETS, 16K AND THE PDGF-RECEPTOR.
Jason Sparkowski, Joanna Anders, Debra Koval and Richard Schlegel. Department of Pathology, Georgetown University, Washington, DC 20007.

The major transforming protein of bovine papillomavirus type 1 (BPV-1), E5, is a strongly hydrophobic 44 amino-acid polypeptide. It mediates the tumorigenic conversion of immortalized rodent cells via a mechanism apparently involving transmembrane interactions with both the 16K membrane pore protein (a component of the vacuolar H^+-ATPase) as well as growth factor receptors, including the PDGF-receptor. E5 contains two domains: a hydrophobic N-terminus responsible for membrane localization and a hydrophilic C-terminus containing two cysteine residues necessary for biological activity and homodimer formation.

A transmembrane glutamine residue in the hydrophobic domain of E5 is strongly conserved in the fibropapillomaviruses (BPV-1, BPV-2, DPV, and EEPV) and limited mutagenesis of this residue has indicated that glutamine is important for biological function and 16K binding. In the current study, we have performed extensive site-specific mutagenesis corresponding to this position and converted it into polar, non-polar, acidic, and basic residues in order to evaluate the role of this glutamine in E5 transforming activity and binding to the 16K protein and PDGF-receptor. All mutant proteins were found to be biochemically stable and were expressed to similar levels in Cos cells. These proteins all formed homodimers and localized normally to cellular membranes. The mutants could be classified into three groups based on their biological activity in C127 cells as compared to wild-type: those with higher activity, those with lower or equal activity, and those whose activities have been abolished. Similar results were also observed in NIH3T3 cells. Interestingly, there was no correlation between the ability of E5 mutant proteins to bind either 16K or the PDGF-receptor and their ability to transform cells. These findings suggest that the E5 glutamine17 residue, while contributing to interactions with cell targets, may also play a critical role in regulating an E5 conformation essential for biological function.

NZ 234 MAPPING THE SEQUENCE-SPECIFIC DNA BINDING DOMAIN OF THE p53 TUMOR SUPPRESSOR PROTEIN, Elena S. Stavridi and Thanos D. Halazonetis, Department of Molecular Oncology, The Wistar Institute, Philadelphia, PA 19104

The p53 tumor suppressor protein complexes with viral proteins, such as SV40 large T antigen, papillomavirus E6 and adenovirus E1b. As a first effort in understanding these interactions, we have mapped the functional domains of wild-type p53.

We used a p53/leucine zipper fusion protein to map the DNA binding domain. Deletions N-terminal to residue 90 or C-terminal to residue 286 did not abolish DNA binding. In contrast, all generated interstitial deletions within residues 90-286 did. Analysis of conservative single amino acid substitutions within this region pointed to residues Arg245, Arg246, Arg277 and Glu278 participating in the p53/DNA interface. These results together with published results by other investigators suggest that p53 has three functional domains: a sequence-specific DNA binding domain (residues 90-286), an oligomerization domain (residues 287-390) and a transactivation domain (residues 1-89). Only the DNA binding domain is known by antibody analysis to adopt distinct conformations between wild-type and mutant p53. Thus for viral proteins, such as large T antigen and E6, that complex only with wild-type p53, interaction might be mediated through the DNA binding domain. In a collaborative effort we are now analysing p53 mutants for interaction with papillomavirus E6.

NZ 235 CHARACTERIZATION OF A TUMORIGENIC HPV 16 IMMORTALIZED HUMAN KERATINOCYTE CELL LINE, Luisa L. Villa and Katia B.L. Vieira, Ludwig Institute for Cancer Research, São Paulo, SP, Brasil.

Human papillomavirus types associated with cervical cancer can immortalize normal human epithelial cells in vitro. This phenotype can be attributed to the expression of E6 and E7 oncoproteins which interfere with cell growth by thorugh their association with the cellular tumor suppressor gene products p53 and pRB. However, only in rare cases the HPV-immortalized cells become tumorigenic suggesting that additional cellular changes are necessary to induce the malignant phenotype. We have originated several immortalized cell lines by transfection with HPV 16 and HPV 18 DNAs extracted from genital tumors (Villa and Schlegel, Virology 181, 1991). One of these cell lines, HF 16.26, was able to induce squamous cell carcinomas in nude mice. It was characterized by kariotype analysis to be an hipertriploid cell lines, with several chromosome alterations. The levels of p53 protein in this cell line are considerably higher than the very low or undetectable levels of this protein in other HPV-immortalized cell lines. Recently, we have been able to establish a cell line derived from the tumor generated in nude mice. Comparison of tumorigenic and non tumorigenic HPV transfected cell lines can contribute to define genetic alterations that are relevant to cell transformation. Such alterations are likely to be similarly involved in the process of cervical carcinogenis in vivo.

NZ 236DEVELOPMENT OF AN EPIDERMAL TRANSGENIC MOUSE MODEL TO STUDY HPV18 INDUCED CARCINOGENESIS.

Xiao-Jing Wang, David A. Greenhalgh, Joshua N. Eckhardt, Donnie S. Bundman, Mary Ann Longley, Joseph A. Rothnagel, *Richard Schlegel and Dennis R.Roop. Departments of Cell Biology and Dermatology, Baylor College of Medicine, Houston, TX 77030; *Department of Pathology, Georgetown University Medical School, Washington, D.C.

In order to create a transgenic model for human papilloma virus (HPV) associated carcinogenesis, we have employed the regulatory elements of a human keratin 1 (HK1) gene to target the expression of the E6 and E7 oncogenes of HPV-18 exclusively in the epidermis. All HK1.E6/E7 expressors were viable, lived normal lifetimes, and older mice (>1 year) possessed numerous small lesions with a verrucous (wart-like) histotype. Analysis of newborn epidermis and lesions revealed that the HPV18.E6/E7 genes were being expressed, with a predominance of the E6*/E7 transcript over the full length E6/E7 message. Therefore, the long latency in lesion appearance may reflect the absence of intact E6 transcripts and the requirement for additional genetic or epigenetic events, before production of an overt lesion. In agreement with this proposal, spontaneous papillomas developed that expressed an activated ras^{Ha} ($A\text{-}T^{61}$, $G\text{-}T^{13}$) oncogene. All lesions expressed keratin genes K1, K6 and K13 in a fashion characteristic of hyperproliferative or benign tumors, with no evidence of malignant conversion. Our results demonstrate that the mouse epidermis represents a relevant in vivo model system to analyze the interaction between HPV and cellular genes in neoplasia.

NZ 237 ROLE OF THE CYCLINS WHICH ARE TARGETS FOR ADENOVIRUS-E1A TRANSFORMATION.

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Transformation of cells will be accompanied by disruption of the normal cell cycle regulation. Adenovirus E1A proteins function by association towards cellular proteins, like the Rb protein and the p107-cyclin A-cdk2 complex. In this manner E1A proteins will disrupt the cdk2 kinase from its normal cellular substrates. One is the substates of the cyclin A-cdk2 was shown to be p107, which *in vitro* was phosphorylated at most of the sites that are also phosphorylated in human cells. Using p107 and histone H1 as substrates, we have found that although the cdk subunit provides a necessary component for binding, it is the cyclin subunit that plays the critical role in targeting a cyclin-cdk complex to p107. Studies whether p107 functions as a bridging factor to bring the cyclin A-cdk2 kinase in close proximity of E2F, showed that in insect cells the cyclin A-cdk2 kinase efficiently phosphorylates E2F1 on sites that are also used in human cells. However, the presence of p107 did not affect the efficiency of this event.

Additional effects of the E1A proteins are the increased expression of cyclin A and the decreased expression of cyclin D1 in Ad-transformed cells. To investigate the role of cyclin D1 repression for the transformed phenotype and to study the role of cyclin D1 in cell cycle regulation, the cyclin D1 cDNA was stably introduced into Ad5-transformed cell lines. The introduced cyclin D1 protein was shown to be complexed with the endogenous cdk4. Sofar, we did not found, in extracts made from exponentially growing cells, an alteration in the phosphorylation state of the Rb protein. In some of the cell lines we observe a severely reduced growth potential and extensive cell death, suggesting that either high levels of cyclin D1 or its deregulated expression during the cell cycle can be lethal to cells. Alternatively, co-expression of E1A and cyclin D1 may be responsible for the observed cell death. Repression of cyclin D1 may therefore be required for cell transformation by 5E1A.

NZ 238 ISOLATION AND CHARACTERIZATION OF YEAST MUTANTS DEPENDENT ON ADENOVIRUS E1A FOR GROWTH,

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The protein products of the adenoviral E1A gene are implicated in a variety of transcriptional and cell cycle events. This involves interactions with several proteins present in human cells, including parts of the transcriptional machinery and negative regulators of cell division such as the Rb gene product and p107. The sites of interaction with cellular proteins have been mapped to several distinct domains within E1A. In order to better understand the activities of E1A and its interactions with other proteins, we have isolated six *Saccharomyces cerevisiae* mutants that depend on expression of the 12S or 13S cDNAs of E1A for growth. Isolation of the mutants is based on a colony color sectoring assay which allows the identification of plasmid-dependent mutants. A plasmid shuffle assay shows that the plasmid dependent phenotype is due to the presence of the E1A cDNAs and that both the 12S and the 13S form of E1A can rescue growth of all mutants equally well. The six mutants fall into three classes that were named *web-1, 2, 3* (wants E1A bad). The phenotypes of mutants in both the *web1* and *web2* groups are due to a single gene defect, and the yeast genes that fully complement the mutant phenotypes of both groups were cloned. Deletion of either *WEB1* or *WEB2* is lethal. Expression of E1A is not able to rescue the lethality of either the *web1* or the *web2* null alleles, implying allele-specific mutations that lead to E1A dependence. The *WEB1* gene sequence encodes a 1273 amino acid protein that is unlike any protein in the database. Tetrad analysis has shown that plasmid dependence in *web1* mutants is linked to the *WEB1* gene, supporting the idea that mutations in *WEB1* directly cause E1A dependence. Plasmid shuffle assays with mutant forms of E1A show that CR1 is required for rescue of the growth of all six E1A-dependent yeast mutants, while the N-terminal 22 amino acids as well as CR2 and the C-terminus are dispensable. Current experiments are aimed at isolating the mutant alleles of the *WEB1* gene, as well as working with multicopy suppressors of the *web1* mutants.

EBV, Lymphocyte Proliferation, and the Viral Immune Response

NZ 300 EBV NUCLEAR ANTIGEN (EBNA) 3C/6 EXPRESSION ABROGATES THE REPRESSION OF LMP1 IN G1 - ARRESTED RAJI CELLS, Martin J. Allday[1,2], Mark Bain[1] and Paul J. Farrell[1], Ludwig Institute for Cancer Research[1] and Department of Medicine[2], St. Mary's Hospital Medical School London, W2 1PG, U.K.

The EBV in the BL line Raji has a deletion in the EBNA3C gene. When Raji cells become growth arrested in the G1 phase of the cell cycle, the level of detectable latent membrane protein - 1 (LMP1) is substantially reduced. After dilution of the cells with fresh growth medium, by 12 hours LMP1 is expressed at a high level. Here we show that in Raji cells which constitutively express a transfected EBNA3C gene the down-regulation of LMP-1 in the growth arrested cells does not take place. We show that in wild-type Raji low level LMP1 expression occurs when most of the cells are arrested at a point(s) in G1 (or G0) when the product of the retinoblastoma gene, Rb p105, is hypophosphorylated. The dramatic synthesis of LMP1 coincides with the progression of these cells to late G1 when Rb p105 becomes hyperphosphorylated. Thus, in Raji, LMP1 gene expression is apparently regulated in a cell cycle-dependent manner but when EBNA3C is present it is constitutive as it is in a lymphoblastoid cell line immortalised by EBV. Furthermore we show that *in vitro* EBNA3C binds to both Rb p105 and the conserved carboxyl terminal end of TBP/TFIID. We are currently determining how these phenomena relate to the regulation of LMP1 and to the essential role of EBNA3C in immortalisation of B cells by EBV.

NZ 301 EBV - RELATED LYMPHOPROLIFERATIVE DISEASE IN THE PEDIATRIC POPULATION, Jane E. Armes, Simone C. Eades, Bruce Ross, Melissa C. Southey, Chung Wo Chow and Deon J. Venter, Division of Pathology, Royal Children's Hospital, Flemington Road, Parkville, Victoria, 3052, Australia.

EBV induces long - term latent infection in B - lymphocytes. Proliferation of these infected lymphocytes is controlled primarily by cell - mediated immune responses. EBV - induced lymphoproliferative disease (LPD) manifests by tumorous deposits of polymorphous lymphocytes at multiple sites and occurs in hosts with deficient cell - mediated immunity, most commonly in the context of HIV infection, organ transplantation or well - defined congenital immunodeficiency states such as X - linked Lymphoproliferative Disorder, Ataxia - Telangiectasia and Wiscott - Aldrich Syndrome. We have studied a patient with congenital anemia and neutropenia, with no documented deficiency in cell - mediated immunity, who died following an EBV - induced LPD. Analysis of IgH chain gene rearrangement from multiple individual lymphoid deposits following PCR amplification of archival and fresh autopsy - derived material showed expansion of different lymphoid clones at different sites. We hypothesise that this patient had defective function of haematopoietic growth factors leading to congenital anaemia and neutropenia. It is now known that such factors (e.g. IL-3, GM-CSF and G-CSF) are directly involved in regulating the survival and function of cells involved in the cell - mediated immune response, and also enhance the production of cytokines by these cells. Hence, in this case, a primary deficiency in cytokine production may be responsible for inefficient cell - mediated attack on EBV - infected lymphocytes, resulting in EBV - induced LPD. This case is being investigated further at the level of the cytokine gene structure and expression, and the results will be presented.

NZ 302 REGULATED EXPRESSION OF THE HERPESVIRUS SAIMIRI ONCOPROTEIN STP-C488 IN TRANSFORMED HUMAN T CELL LINES, Brigitte Biesinger, Helmut Fickenscher, Andrea Knappe, Sabine Wittmann, and Bernhard Fleckenstein, Institut für Klinische und Molekulare Virologie der Universität Erlangen-Nürnberg, Loschgestr. 7, D-91054 Erlangen

Herpesvirus saimiri has long been known as a T cell transforming virus of New World primates. As we had demonstrated, virus strains of subgroup C are also capable of transforming human T lymphocytes to permanent growth in vitro. They express the phenotype of mature activated T cells growing independently of restimulation either by mitogen or antigen. In response to stimulation via the CD3 and CD4 molecules they behave like normal activated cells with respect to protein tyrosine phosphorylation and calcium mobilisation. When antigen-specific T cell clones are transformed by the virus they retain their antigen specificity. In contrast to New World Monkey lymphocytes the human T cell lines do not produce detectable amounts of virus after transformation. In the human cells only one virus-specific transcript has been identified which is encoded by the transformation associated part of the viral genome. Transcription is not dependent on de novo protein synthesis as high levels of mRNA are found after cycloheximide treatment. Stimulation by the phorbolester TPA dramatically augments basal expression. Thus, regulation of transcription seems to be similar to early T cell activation genes. One of the two open reading frames encoded by the resulting mRNA has been termed STP-C488 (Herpesvirus saimiri transformation associated protein of strain C488) because the viral gene product had been identified as an oncoprotein in rat-1 fibroblasts and in transgenic mice. In the transformed human T-lymphocytes STP-C488 is abundantly expressed in the cytoplasm where it might influence signalling cascades leading to activation independent growth.

NZ 303 IDENTIFICATION OF A MURINE T-HELPER CELL EPITOPE ON THE MAJOR (L1) CAPSID PROTEIN OF HUMAN PAPILLOMAVIRUS TYPE 16 AND ITS UTILIZATION TO POTENTIATE SERUM AND SECRETORY ANTIBODY RESPONSES TO A B-CELL EPITOPE. John Cason, Parminder Kambo, Georgina Siggers, Richard J. Jewers and Jennifer M. Best. The Richard Dimbleby Laboratory of Cancer Virology, The Rayne Institute, St Thomas' Hospital, U.M.D.S., London SE1 7EH,U.K.

There is at present considerable interest in developing vaccines against HPV-16[1]. We have investigated the possibility of developing synthetic prophylactic vaccines containing T-helper and B-cell epitopes of the L1 protein. A promiscuous T-helper cell epitope on L1 was identified (aa. 269-284) in lymphocyte transformation assays of cells from five strains of mice. Peptides encoding the T-helper epitope were then synthesized with a well characterized L1 B-cell epitope (aa. 193-218 which is recognized by the mAb Camvir-1) attached in various positions (at COOH or NH2 or at both termini: i.e. T-B, B-T, B-T-B). Mice immunized with these constructs (so that all received an equimolar dose of the B-cell motif) responded to produce high titre, high avidity, IgG antibodies only when the B-T-B construct was used. The B-T-B construct was also able to stimulate the production of secretory antibodies present in faeces and vaginal but not buccal secretions.

[1]Cason et al., 1993. Vaccine **11**, 603-611.

NZ 304 CHARACTERIZATION OF 5'-UPSTREAM SEQUENCE OF THE LATENT MEMBRANE PROTEIN (LMP1) GENE OF AN EPSTEIN-BARR VIRUS VARIANT, Chen M.L., Wu R.C., Liu S.T. and Chang Y.S., Department of Microbiology and Immunology, Chang-Gung Medical College, Taiwan, R.O.C.

An Epstein-Barr virus (EBV) variant (designated as a NPC strain) was predominantly found in the nasopharyngeal carcinoma tissues in the Taiwanese population. This virus strain contains a more pathogenic latent membrane protein 1 (LMP 1) gene than the B95-8 strain does. Sequence analysis revealed that variations between two strains occurred in both 5'-upstream sequence and the gene coding region. In this work, we use the chloramphenicol acetyltransferase (CAT) gene as a reporter gene to find out if these variations will have any effect on the promoter activity of the 5'-upstream region of LMP 1 gene. Data showed that the sequence between -54 and +20 of these two strains contained basic, constitutive promoter activity. Therefore, the sequence was defined as the minimal promoter region. We also identified two other regions between -950 and -495, and -223 and -191, respectively, that showed an inhibitory effect on the basic, constitutive promoter activity. Furthermore, the CAT activities derived from 5'-upstream regions were up-regulated by EBV-encoded nuclear antigen 2 (EBNA 2) in B lymphocytes, but not in an epithelial cell line, C-33A. Results also indicated that the promoter activity of the NPC strain constantly showed a three-fold difference from that of the B95-8 strain. The sequence variations between -54 and +20 of both strains indicated that an activating transcription factor (ATF) binding site, tgacgtag, was present in B95-8 strain, however, was changed to tctcgtag in the NPC strain. Substitution of the B95-8 sequence with the NPC sequence caused the CAT activity of B95-8 strain to drop to the level of the NPC strain. On the other hand, the exchange of the NPC sequence with the B95-8 sequence restored the CAT activity. This suggested that the sequence variations in ATF binding site may contribute to the higher CAT activity observed in the B95-8 strain. The biological significance of this difference is referred to in the discussion.

NZ 305 **IN VIVO EXPRESSION AND LOCALISATION OF BHRF-1, AN EPSTEIN BARR VIRUS (EBV) ENCODED BCL-2 HOMOLOGUE, Paul Clarke, Tamas Hickish, David Robertson, Mark Hill, Francesca di Stefano and David Cunningham, CRC Section of Medicine, Lymphoma unit and Department of Cell Biology, Institute of Cancer Research and Royal Marsden Hospital, Sutton, Surrey, United Kingdom.**

BHRF1 is abundantly expressed early in the EBV replicative cycle and transiently in most EBV infected cell lines following serum deprivation. The BHRF1 protein has significant sequence similarity with the proto-oncogene bcl-2 that has been implicated in the development of human B cell follicular lymphoma. Bcl-2 is a regulator of programmed cell death that has been proposed to function via its association with the mitochondrial membrane. Recently a number of bcl-2 related proteins have been identified. Sequence alignment of BHRF1 with these proteins reveals that the major regions of similarity between BHRF1 and bcl-2 are conserved throughout this family implying that BHRF1 is indeed a bcl-like protein. We have investigated the localisation of BHRF1 using immuno-electron microscopy combined with a fixing technique that maintains cell integrity. In EBV infected cell lines the majority of BHRF1 localises to the mitochondrial membrane in a manner similar to bcl-2. To further investigate the possible functional similarity between BHRF1 and bcl-2 we have established a colony of transgenic mice that are constitutively overexpressing BHRF1 from an Ig heavy chain promoter element. These mice are now being monitored for the development of follicular hyperplasia, which may eventually transform to lymphoma. Such an observation would be reminiscent of the phenotype displayed by transgenic mice overexpressing bcl-2 and would imply that bcl-2 and BHRF1 are functionally or mechanistically related. Preliminary analysis suggests that this is indeed the case.

NZ 306 ACTIVATION OF NATURAL PROMOTER ELEMENTS BY ZEBRA, THE EPSTEIN-BARR VIRAL LYTIC TRANSACTIVATOR, IN *SACCHAROMYCES CEREVISIAE*, Jill K. Countryman, Lee Heston, Lyn Gradoville and George Miller, Dept. of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510

The latent cycle of Epstein-Barr Virus is disrupted upon expression of the protein product from the BZLF1 open reading frame. This protein, .ermed ZEBRA, initiates a cascade of events culminating in the release of mature viral particles. ZEBRA shows structural homology to jun/fos, and binds degenerate AP-1 like sequences. Inducible expression of ZEBRA in *Saccharomyces cerevisiae* has permitted the analysis of activation of downstream targets by ZEBRA. Our experiments show that ZEBRA will activate both synthetic and natural viral targets in yeast. Using this system, we have identified two different classes of ZEBRA responsive promoters. One class is exemplified by the promoter for BZLF1(Zp) itself. Our results indicate that Zp is activated 5-fold by ZEBRA, and is also activated by endogenous yeast factors in the absence of ZEBRA. The promoter for the BMRF1 gene, EAp, is activated 50-fold in the presence of ZEBRA, but is inactive in the absence of ZEBRA. Analogous experiments performed in B-cells indicate that Zp is also spontaneously activated without ZEBRA expression. Thus, the yeast system may help in the identification of cellular factors which interact with ZEBRA, as well as permitting the identification of novel ZEBRA response elements.

NZ 307 SELECTIVE MUTATIONS IN THE ANCHORS OF TWO HLA A11 RESTRICTED CTL EPITOPES IN EPSTEIN-BARR VIRUS STRAINS FROM HIGHLY HLA A11 POSITIVE POPULATIONS.

Pedro O.de Campos-Lima, Victor Levitsky, Jill Brooks, Steve Lee, Alan B. Rickinson, and Maria G. Masucci. Microbiology and Tumorbiology Center, Karolinska Institute, 177 71 Stockholm, Sweden.

We have previously shown that EBV isolates from areas of Papua New Guinea where HLA-A11 occurs in relatively high frequencies carry a point mutation, affecting the immunodominant HLA A11 restricted CTL epitope in EBNA4 The lys residue in position 424 (corresponding to the anchor position 9 of the CTL epitope) was substituted by thr in all six New Guinea isolates studied. We have now extended this analysis to EBV strains derived from a second highly A11-positive population, Southern Chinese (A11 frequency between 45-67%). All 23 Chinese EBV isolates tested carried mutations affection the immunodominant 416-429 EBNA4 epitope. About half of the isolates carried additional mutations affecting a second A11 restricted CTL epitope recently identified in residues 399-408 of the EBNA4 protein. Mutations within the 426-424 epitope selectively affected the anchor residues in position 2 and 9 with val in position 2 being substituted by a leu (L2 mutation) or lys in position 9 being substituted by either thr, asp, or arg (T9, N9, R9 mutations). HLA A11 positive LCLs transformed by EBV strains carrying these mutations were not recognized by CTL clones specific for the B95.8 epitope. Furthermore, three A11-positive Chinese donors infected by these viruses were unable to mount A11-restricted EBV specific CTL responses. EBNA4 is only known to be expressed in the virally transformed immunoblasts, our finding therefore suggests that a weakening of the EBV-immunoblast rejection response may provide the virus with a survival advantage.

NZ 308A TRANSGENIC MOUSE MODEL OF THE IMMUNOBIOLOGY OF HPV16 INDUCED CERVICAL CANCER. Frazer,I.H.[1], Lieppe,D.[2], Lambert,P.[2], Griep,A.[2] Dunn,L.[1] Tindle, R.W.[1], Fernando,G.[1] [1]Papillomavirus Research Unit, University of Queensland, Australia and [2]McArdle Laboratory for Cancer Research, University of Wisconsin, Madison,Wi. Ph +61 7 240 2251 Fax +61 7 240 2048

Cervical cancer frequently arises from HPV16 infected cervical epithelium, and HPV16+ve cervical cancer cells generally express HPV16 E6 and E7 ORF proteins. Patients with HPV16 infection make little antibody to the E7 protein, whereas 20%-50% of patients with invasive HPV16+ve cervical cancer demostrate humoral immunity to E7. A line(#19) of FVB derived mice have recently been described which are transgenic for HPV16 E6 and E7 ORFs(1). These mice develop skin disease, and eventually E7 mRNA and protein +ve squamous cancers of skin. To examine the immunologic consequences of E7 expression in these mice we examined humoral immune responses to E7 and to a series of overlapping peptides covering the E7 ORF. Six to twelve week old #19 mice had no skin disease and no measurable serum antibody to E7 protein. They responded to immunisation with E7 by producing antibody to E7, including antibody to previously established immunodominant epitopes at the N-terminal(EYMLD) , and in the middle(QAEPD) of the protein. No significant differences in the response to E7 were seen between the #19 mice and age matched FVB controls. Older #19 mice with skin disease had antibody to E7 not seen in #19 littermate controls. This antibody, in contrast to that which developed following immunisation, recognised predominantly the C terminal peptides of E7. The #19 mice provide a model for E7 protein presentation to the immune system which mimics E7 presentation in natural HPV infection, and should allow study of (i) the immune response to E7 protein as a determinant of outcome of HPV infection and (ii) the role of keratinocytes in antigen presentation.

1) Lambert PF, Pan H, Pitot HC, Liem A, Jackson m, Griep AE: Epidermal cancer associated with expression of human papillomavirus type 16 E6 and E7 oncogenes in the skin of transgenic mice. Proc Natl Acad Sci U S A (1993) 90, 5583-5587

NZ 309ROLE OF EPSTEIN BARR VIRUS IN THE PATHOGENESIS OF BURKITT'S LYMPHOMA: TRANSACTIVATION OF C-MYC PROMOTER AND IG ENHANCERS BY EBNA-1, Jean-Gabriel Judde, Vinay K. Jain and Ian T. Magrath, Lymphoma Biology Section, NCI, NIH, Bethesda, MD 20892.

Epstein Barr virus (EBV), a ubiquitous human herpes virus, is the causative agent of infectious mononucleosis and lymphoproliferative disorders in immunocompromised hosts, and is closely associated with Burkitt's lymphoma (BL), nasopharyngeal carcinoma and AIDS-associated non-Hodgkin's lymphoma (NHL). Despite this association and EBV's ability to immortalize B cells in vitro with high efficiency, its role in the pathogenesis of these tumors remains largely speculative. Whereas the ability of EBV to transform B cells in vitro is dependent on the expression of a battery of some 10 latent genes, the only EBV latent gene consistently expressed in BL is the EBV nuclear antigen-1 (EBNA-1). EBNA-1 is a transactivator that binds to the EBV origin of replication (oriP) and is essential for the maintenance of the EBV episome in the cell. A hallmark of BL is the presence of chromosomal translocations which juxtapose the c-myc proto-oncogene to immunoglobulin (Ig) loci; the resulting deregulation of c-myc expression is thought to play a crucial role in BL pathogenesis. We have examined the possibility that EBNA-1, in addition to its role in the replication of latent EBV, may be involved in the deregulation of c-myc in BL, possibly through an effect on the activity of Ig enhancers juxtaposed to c-myc by virtue of the chromosomal translocation. We performed transient transfection experiments with plasmids containing the human c-myc promoter attached to a reporter gene with or without IgH or Ig3'κ enhancers into EBV negative cell lines or the same cell lines stably transfected with a functional EBNA-1 gene. We found that, depending on the cell line, EBNA-1 could increase transcription from the transfected c-myc promoter alone, and/or transactivate Ig enhancers. Preliminary mapping with 5' deletion mutants of the c-myc promoter suggests that response element(s) located in the first intron, a region known to participate in the regulation of c-myc expression, are at least partly involved in the transactivation by EBNA-1. These studies indicate a new role for EBNA-1 which could be relevant to the pathogenesis of BL and point to a possible interaction between EBNA-1 and cellular proteins resulting in the transactivation of c-myc and Ig enhancers.

NZ 310 A VIRAL-ENCODED CYCLIN HOMOLOG WITH ASSOCIATED PROTEIN KINASE ACTIVITY, Jae U. Jung and Ronald C. Desrosiers, New England Regional Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, MA 01772

Infection of non-human primates with herpesvirus saimiri results in rapidly progressing malignant T cell lymphomas. We have identified within its genome as oncogene, STP, which is not required for viral replication but which is required for in vivo oncogenicity and in vitro transformation. Recent DNA sequence analysis of the entire 113 kbp of the herpesvirus saimiri genome revealed three additional open reading frames which could possibly contribute to transformation by this virus: an open reading frame homologous to cellular G1 cyclins; a homolog of a superantigen (SH); and a homolog of cellular G-protein coupled receptor (G-PCRH). The protein encoded by the *eclf2* gene of this oncogenic herpesvirus was found to have an apparent molecular size of 29 kDa in transformed cells and was phosphorylated *in vivo*. Immunofluorescence staining showed that eclf2 protein was localized to the nucleus during the G1/S phase and it accumulated in the cytoplasm during other phases of the cell cycle. Eclf2 protein in mammalian and insect cells was found to be associated with potent protein kinase activity. However, we failed to demonstrate any association with specific protein kinases that are known to associate with cellular cyclins. Eclf2 was labeled by radioactive analogs of ATP, suggesting that eclf2 itself may be directly responsible for the kinase activity. Eclf2-kinase can phosphorylate eclf2 and other protein substrates including histone H1 and Rb fusion protein in vitro. Thus, eclf2 resembles cellular cyclin D in primary sequence, in its cell cycle-dependent cellular localization, and in the presence of associated protein kinase activity. However, unlike cellular cyclins, eclf2 has intrinsic ATP-binding activity and thus may itself be a protein kinase.

NZ 311EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 2 FUNCTION IS REQUIRED FOR MAINTENANCE OF EPSTEIN-BARR VIRUS INDUCED B-CELL TRANSFORMATION. Bettina Kempkes[1]*, Henri-Jacques Delecluse[1], Christine Rottenberger[1], Elisabeth Kremmer[2], Georg W. Bornkamm[1] and Wolfgang Hammerschmidt[1], GSF - Forschungszentrum für Umwelt und Gesundheit [1] Institut für klinische Molekularbiologie und Tumorgenetik, [2] Institut für Immunologie, 81377 München, Germany

Infection of primary B-lymphocytes by Epstein-Barr Virus (EBV) leads to growth transformation of these B-cells in vitro. Epstein-Barr Virus nuclear antigen 2 (EBNA2) is one of several latent gene products which are essential for growth transformation of primary B-lymphocytes by EBV. EBNA2 is one of the first genes expressed after EBV infection of B-cells. As a transcriptional enhancer of viral and cellular genes it transactivates the latent membrane protein (LMP) genes and induces the cell surface markers CD21 and CD23 in EBV negative B-cell lymphoma lines. We generated conditional EBNA2 mutants by expressing EBNA2 as fusion protein of the hormone binding domain of the estrogen receptor. Growth transformation of primary B-cells which express exclusively the conditional EBNA2 mutants resulted in estrogen dependent lymphoblastoid cell lines. These cell lines undergo multiple phenotypic alterations including growth arrest and termination of DNA synthesis after removal of estrogen from the culture medium. Growth arrest in the absence of estrogen is reversible and DNA synthesis can be rescued by readding estrogen to the system.

NZ 312 THE INFLUENCE OF EBV ON THE REGULATION OF THE IL10 PROMOTER IN BURKITT LYMPHOMAS,

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Cytokines may regulate the growth and differentiation of normal hematopoietic cells and are possibly involved in the biology of malignant lymphoma and leukemia. Epstein-Barr virus (EBV) can cause B-lymphoproliferative disorders in immunocompromised human.
To analyze the expression of cytokines in Burkitt's lymphoma cell lines (BL) and Chronic lymphatic leukemia (CLL) we have used the RT-PCR. The results are summerized in the table showing the EBV dependent expression of IL10 and TNF-ß in BL:

		IL4	IL7	IL10	IL11	TNF-ß
BL	EBV neg	0/5	0/5	0/5	4/5	0/5
	EBV pos	5/7	0/7	8/8	4/7	8/8
	B95-8 hyperinf.	2/3	2/3	3/3	0/3	3/3
	P3HRI hyperinf.	1/3	0/3	0/3	1/3	0/3
LCL		5/6	2/6	5/6	4/6	6/6
CLL	EBV neg		10/10	8/10		
PBL.		-	+/-	-	-	-

Thus IL10 is predominantly expressed in EBV positive BL and maybe involved in immunsuppressive pathways in BL patients. We have analyzed a 1300bp fragment of the IL10 promoter, to find out sequences within the promoter which are activated by specific EBV genes. Using a luciferase reporter system we can show that only in EBV wild type (B95-8) containing B-cells the 1300bp fragment is active, but not in P3HRI infected BL. The EBNA2 gene is therefore involved in IL10 transcriptional regulation. The main promoter activity is located within the first 250bp downstream from the translation start of IL10 in BL. An additional activation was found by deleting a 360bp fragment between -700/-340bp .
Our datas demonstrate that EBV can activate IL10 transcription and additional factors maybe involved in IL10 production in EBV negative B-cell malignancies, like CLL.

NZ 313 DIFFERENT IMMUNOGENICITY OF TWO HLA A11 RESTRICTED CTL EPITOPES IN THE EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN-4.

Victor Levitsky, Qian-Jin Zhang, Teresa Frisan, Pedro O. de Campos-Lima and Maria G. Masucci. Microbiology and Tumorbiology Center, Karolinska Institute, 177 71 Stockholm, Sweden.

We have previously show that the EBV nuclear antigen EBNA4 is the major target of HLA A11 restricted EBV specific CTL generated by in vitro stimulation of lymphocytes from EBV-seropositive donors with autologous virus infected cells. Screening with vaccinia vectors encoding progressively truncated forms of EBNA4 and peptide sensitization tests, using overlapping 14 or 15 AA synthetic peptides covering the entire 938 AA EBNA4 sequence, have allowed identification of 5 epitope regions between residues 101-115, 396-410, 416-429, 481-495, and 551-564. CTLs from 10 out of 12 A11-positive donors tested reacted against the 416-429 peptide. In addition, most of the donors showed detectable CTL responses to the 396-410 epitope whereas responses against the other epitopes were less strong or were not detected in some of the donors. The nomaner 416-424 (IVTDFSVIK) and the tenmer 399-408 (AVFDRKSVAK) have been identified as the cognate peptides. CTL precursors specific for these peptide epitopes are not cross-reactive and are present at different frequencies in the T-cell pool of healthy A11-positive EBV carriers. HLA A11 positive LCLs transformed with the B95.8 EBV strain are more sensitive to lysis by CTLs specific for the 416-424 epitope, as a rule. This is not due to different efficiency of the effectors since CTL clones specific for the 426-424 and 399-408 epitopes lyse equally well PHA blasts preincubated with the corresponding synthetic peptides. HLA class I stabilization tests using an A11-transfected subline of the transporter mutant cell line T2 show that the 416-424 peptide binds with higher affinity to empty A11 molecules. The results suggest that the immunogenicity of individual epitopes may be influenced by their representation within the cell surface pool of A11/peptide complexes.

NZ 314 USE OF A DOUBLE ARO SALMONELLA MUTANT TO STABLY EXPRESS HPV16 E7 PROTEIN EPITOPES CARRIED BY THE HBV CORE ANTIGEN, Patricia Londono (1), Steve Chatfield (2), Robert Tindle (3), Ian Frazer (3) and Gordon Dougan (1), Department of Biochemistry, Imperial College. London. UK (1). Vaccine Research Unit. Mediva Group Research. Imperial College London. U.K (2). Lions Immunology Laboratory. Princess Alexandra Hospital. Brisbane . Australia (3).
Attenuated salmonella strains have been used as vectors for delivering antigens from other pathogens via the oral route. These organisms are particularly efficient in eliciting mucosal responses as well as concomitant humoral and cellular responses against the heterologous antigen. We have designed a plasmid named pGA in which the full-length HBV core antigen (HBcAg) has been cloned under control of the nirB promoter, a promoter induced by anaerobiosis. An attenuated double aro *Salmonella typhimurium* mutant strain transformed with pGA is able to express high levels of HBcAg antigen in anaerobic conditions, has proved to be stable *in vivo* and induces the production of antibody responses against the core antigen in mice immunised on two occasions by the oral route. Since the HBcAg has been successfully used in the past as a carrier to induce immune responses against inserted viral epitopes, we have used plasmid pGA to express immunodominant sequences from the E7 protein of the human papillomavirus type 16 (HPV16) in the vaccine strain *Salmonella typhimurium* aroA aroD mutant. A peptide sequence containing B and T epitopes of the E7 protein has been inserted in the e1 loop of HBcAg. The resulting hybrid salmonella strain has been used to immunise mice orally and has shown to be stable *in vivo* . After two oral inoculations with the live recombinant strain, no anti-E7 antibodies were detected in the sera of vaccinated Balb/c mice. Nevertheless, a strong serum IgG response against E7 was produced within seven days of inoculating the same mice with the purified E7-HBcAg chimeric product, indicating that the animals were immunologically primed against E7 as a result of vaccination with the recombinant salmonella strain. We are currently testing the ability of this strain to induce cellular responses against the papillomavirus epitopes in Balb/c mice, as well as its ability to induce immunological responses in other strains of mice.

NZ 315 SEQUENCE VARIATION IN EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1

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The Epstein-Barr Virus, a human herpes virus, is an important factor in a number of malignancies including Nasopharyngeal Carcinoma (NPC) and post transplant lymphoma (PTL). Interestingly, the prevalence of NPC is limited geographically, with a higher incidence of NPC in Southern China, Mediterranean Africa, and Alaska. The viral encoded Latent Membrane Protein (LMP1) is expressed in a majority of the NPC and PTL tumors and has been shown to have transforming properties in Rat-1 cells. A predominant strain of EBV marked by an XhoI restriction enzyme polymorphism (REP) within the LMP1 gene has been identified in Type I EBV in NPC from Southern China. This polymorphism was also present in Type II EBV in NPC from Alaska. In this study, the sequence of the LMP1 gene was determined in samples representing Type I and Type II EBV. Consistent nucleotide variation in the amino terminus of LMP1 was identified in both Type 1 and 2 strains marked by the XhoI REP. Eleven changes were present in the NPC isolates, seven of which resulted in amino acid changes. Deletion of amino acids 343-352, corresponding to the B95-8 LMP1 sequence, was detected in ~40% of isolates analyzed. Amino Acids 343-352 were deleted in all of the Chinese isolates analyzed, but were present in the Alaskan isolates. This data indicates that the Chinese and Alaskan isolates are clearly different strains of EBV which have both developed the same mutations in the amino terminus of LMP1. The consistent changes found in these isolates may reflect a functional change in the LMP1 protein or may be due to selective pressure on LMP1 from the immune system. The LMP1 sequences also differed in the number of an 11 amino acid (aa) repeat element. PCR analysis of the latently infected NPC tissue demonstrated the presence of a single number of repeats in contrast to the permissive infection, hairy leukoplakia, which produced multiple bands as a result of recombination during lytic replication of EBV. This suggests that recombination in the LMP1 repeats may occur during multiple rounds of infection in hairy leukoplakia. In summary, this data indicates that a unique strain of EBV containing distinct amino acid changes in LMP1 is prevalent in Chinese and Alaskan populations and supports the previous observation that NPC is a clonal expansion of a singly infected cell.

NZ 316 USE OF RETROVIRAL MEDIATED EXPRESSION OF HBV PRECORE AND CORE ANTIGENS TO INDUCE IMMUNE RESPONSES IN MICE, Joanne O'Dea, Kay Townsend, Stephen Chang, Douglas Jolly, and William T. L. Lee, Department of Viral Therapeutics, Viagene, Inc., 11075 Roselle Street, San Diego, CA 92121

Antigen-specific MHC Class I restricted cytotoxic T lymphocyte (CTL) responses appear to have an important role in clearance of Hepatitis B virus (HBV) during recovery from acute infection. Immunodominant CTL epitopes have been identified within the sequence common to HBV precore and core antigens (Penna et al., J. Exp. Med., 1991, 174:1565-1570). Viagene has previously shown that recombinant retroviral mediated expression of HIV-1 env and HSV-1 gB antigens induce potent and specific MHC Class I restricted CD8+ CTL responses in mice. Therefore, we are investigating the use of recombinant retroviral vectors encoding HBV precore and core antigens to induce specific MHC Class I restricted CTL responses in mice. The long term goal is to use retroviral mediated expression of HBV antigens as a therapeutic for treatment of individuals chronically infected with Hepatitis B virus.
Replication defective recombinant retroviral vectors encoding HBV precore antigen and core antigen were used to transduce the mouse fibroblast, LMTK-. Expression of precore and core antigens in the transduced cells was analyzed by ELISA and Western blot. Replication defective recombinant retroviral vector, expressing either HBV precore/e or core, was injected intraperitoneally and/or intramuscularly into syngeneic C3H (H-2^k) mice. Splenocytes from immunized mice were harvested and restimulated *in vitro* with LMTK- transduced fibroblasts expressing either precore or core antigen. The restimulated effectors kill targets expressing the precore/e antigen. We are currently determining the specificity of this response by testing the effectors on peptide coated targets.

NZ 317 EXPRESSION OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 IN TRANSGENIC MICE. Nancy Raab-Traub, Wanla Kulwichit, and Rachel Hood Edwards. Dept. of Microbiology and Immunology, and the Lineberger Comprehensive Cancer Center, The University of North Carolina, Chapel Hill, NC 27599

The Epstein-Barr virus (EBV) is closely associated with human malignancies which develop in both lymphoid and epithelial cells and efficiently transforms B-lymphocytes *in vitro*. In nasopharyngeal carcinoma (NPC), parotid carcinoma, and post-transplant lymphomas, the EBV latent membrane protein, LMP1, is consistently expressed. LMP1 has transforming properties in rodent cell lines and induces activation markers in established B-cell lines. To determine if LMP1 expression directly alters cellular growth properties and contributes to oncogenesis, transgenic mice lineages were established containing the LMP1 under the control of the immunoglobulin promoter/enhancer (IG) and the K14 keratin promoter.

Four IG-LMP1 lineages have been established. Lymphoid malignancies developed in three of the founders with expression of the transgene detected at the RNA and protein level in the tumor tissues. In animals without overt pathology, LMP1 expression was detected in salivary gland, spleen, and lymph node. Histopathologic examination of the spleens suggest increased cellularity while increased levels of lymphoblasts were detected in lymph nodes. These data suggest that LMP1 can induce lymphoid hyperplasia, a situation perhaps analogous to an expanded premalignant population. Moreover the generation LMP1 positive lymphomas in all of the old founder mice suggests that clonal lymphomas can emerge from the LMP1 expressing cell population, a process which may mimic malignant progression *in vivo* where clonal lymphomas emerge from initially polyclonal outgrowths.

The K14 keratin promoter functions in basal epithelium. In transgenic lineages expressing LMP1 under the control of the K14 promoter, expression of LMP1 was detected in skin and tongue. In some tissues, expression was associated with abnormal maturation of keratinocytes and elevated keratin formation. The K14 LMP1 transgenic lineages are the first model for the effects of expression EBV latent, transforming genes in normal epithelium.

NZ 318 TRANSCRIPTION OF EPSTEIN-BARR VIRUS BAMHI A AND LMP1 GENES IN NASOPHARYNGEAL CARCINOMA. Robert Sadler and Nancy Raab-Traub,
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Epstein-Barr virus (EBV) is consistently detected in nasopharyngeal carcinoma (NPC), and in other human malignancies, and is likely to play a causative role in tumorigenesis. In NPC, EBV gene expression is restricted to a small number of genes including the BamHI A family of transcripts and Latent Membrane Protein 1 (LMP1).

Transcription through the BamHI A region of EBV is abundantly detected in NPC, and consists of highly-spliced messages ranging in size from 4.0 to 7.6 kb. Partial BamHI A cDNA's previously isolated contain distinct splicing patterns, but all include a 525 bp ORF, BamHI A rightward frame 0 (BARF0). Three additional BamHI A cDNA's, all of which have distinct splicing patterns and contain the BARF0 ORF, are presented here. PCR analysis of size-selected mRNA was performed to assign the different exon patterns of these cDNA's to the differently-sized bands detected by Northern blotting. The data indicate that these bands do not contain singular species of transcripts but rather consist of heterogeneous populations of messages. 3' RACE analysis has revealed that these messages are also heterogeneous at their 3' ends. Cleavage of primary BamHI A transcripts gives rise to two classes of messages: those that encode the complete BARF0 ORF and those that are slightly truncated and do not include the BARF0 stop codon. This processing probably involves the differential function of the two poly-A signals at the 3' end of the ORF, and could provide a novel regulatory mechanism for the expression of this gene.

EBV LMP1 is encoded by messages of 2.8 and 3.7 kb which are 3' co-terminal and identically spliced through the coding region. A previously isolated partial cDNA representing the larger message extended 199 bp upstream of the smaller transcript. In this study the 5' sequence of the 3.7 kb transcript has been further defined by cDNA cloning, RNAse protection, and Northern blotting. The transcript is unique in that it contains sequence from the EBV terminal repeats and may initiate in this region.

NZ 319 THE EPSTEIN-BARR VIRUS ENCODED MEMBRANE PROTEIN LMP1 INDUCES INTERLEUKIN-10 PRODUCTION IN BURKITT'S LYMPHOMA LINES. Jan Sjöberg, Hiroshi Nakagomi, Riccardo Dolcetti, Maria Teresa Bejarano, Pavel Pisa, Rolf Kiessling and Maria G. Masucci, Microbiology and Tumorbiology Center, Karolinska Institute, 177 71 Stockholm, Sweden.

Human interleukin-10 (h-IL-10) is a pleiotropic cytokine with stimulatory activity on B-lymphocytes. Recent evidence indicates that infection with Epstein-Barr virus (EBV) induces h-IL-10 production in B-cells and this cytokine may contribute to EBV-induced B-cell transformation. It is not known whether h-IL-10 induction by EBV correlates with distinct phenotypic features of the infected cells or with the expression of particular viral genes. We have approached these questions by investigating the expression of h-IL-10 mRNA in a panel of B-cell lines including: in vitro EBV transformed lymphoblastoid cell lines (LCLs), EBV carrying Burkitt's lymphoma (BL) lines, EBV negative BL lines and their sublines infected with different EBV strains, or transfected with the transformation-associated viral gene. h-IL-10 mRNA was detected by reverse transcriptase assisted (RT)-PCR in a subset of EBV negative BLs and in all EBV positive BL lines and LCLs investigated except Daudi. This cell line carries an EBNA2 gene defective virus strain. h-IL-10 mRNA was induced by conversion of three EBV negative and h-IL-10 negative BL lines with the transforming, B95.8 derived, EBV strain. P3HR-1 virus convertants that do not express the viral EBNA2 and the EBV latent membrane protein LMP1, and fail to progress towards a LCL-like cell phenotype, showed no evidence of h-IL-10 up-regulation. Expression of LMP1 was sufficient to induce h-IL-10 mRNA in transfected sublines of the EBV negative DG75 and BL41 cell lines whereas expression of EBNA1, -2, -5 or -6 had no effect. h-IL-10 was detected in the culture supernatants of the LMP1 transfectants by specific ELISA assays. The present findings confirm the role of LMP1 in the transactivation of a wide variety of cellular genes which may be involved in EBV induced B-cell transformation.

NZ 320 EVIDENCE FOR LYTIC INFECTION BY EPSTEIN-BARR VIRUS (EBV) IN MUCOSAL LYMPHOCYTES INSTEAD OF NASOPHARYNGEAL EPITHELIUM IN NORMAL INDIVIDUALS. G.Srivastava, Q.Tao, A.C.L.Chan, S.L.Loke and F.C.S.Ho. Department of Pathology, University of Hong Kong, Queen Mary Hospital Compound, Hong Kong.

Normal nasopharyngeal tissues from six individuals who died of causes unrelated to the respiratory system and had no evidence of EBV-related diseases were studied. EBV-genome was detected by PCR in 5/6 of these cases. Using immunostaining for EBV-proteins in these six cases, we have detected expression of EBV nuclear antigen 2, latent membrane protein, and switch protein ZEBRA in one of the five PCR positive cases in stromal small lymphoid cells but not in epithelial cells. Only scanty LMP+ cells were observed and most of these cells were aggregated in small focal regions under the epithelium while the rest were distributed as isolated cells in the submucosa. ZEBRA and EBNA2 positive cells were less frequent and were randomly scattered. Using a double immunostaining method combining alkaline phosphatase anti-alkaline phosphatase and indirect immunofluorescence, LMP+ cells were found to be either CD19+, or less frequently CD3+, but none were CD68+. These results suggest that both B and T-lymphocytes harbouring EBV can be found in the normal nasopharynx. Interestingly, EBV-proteins associated with lytic viral replication were also detected by immunostaining in this case. Weak granular staining for EBV early antigen, moderate staining for membrane antigen and viral capsid antigen was observed in rare cells in the corresponding region as LMP+ cells, implying lytic infection by EBV of stromal lymphocytes in this case.

These findings suggest that mucosal lymphocytes by themselves appear to be sufficient to support the persistence and replication of EBV without the need of an epithelium to act as a primary reservoir. The results are consistent with the model for the lifelong persistence of EBV in asymptomatic virus carriers in which the lymphocytes have been proposed to serve both as a long-term repository of the viral infection and as a vehicle for delivering the virus to secondary permissive sites *in vivo*.

NZ 321 EPSTEIN-BARR VIRUS RECOMBINATION IN ORAL HAIRY LEUKOPLAKIA. Dennis M. Walling, Ashley G. Perkins and Nancy Raab-Traub. Div. of Infectious Diseases, Dept. of Microbiology and Immunology, and the Lineberger Comprehensive Cancer Center, The University of North Carolina, Chapel Hill, North Carolina 27599 USA.

Oral hairy leukoplakia (HLP) may be coinfected with multiple types and strains of productively replicating EBV. Viral DNA from biopsies of HLP was analyzed at the *Bam*HI YH region and the EBNA-2 gene to characterize the viral strains and recombinant variants of EBV present.

Cosmid cloning of the *Hin*dIII B fragment from a single HLP generated clones containing distinct *Bam*HI H fragments. Sequencing the EBNA-2 gene demonstrated identity between each clone, indicating common origin from a single parental strain. Restriction fragment mapping and partial sequencing of the *Bam*HI H fragments of each clone revealed several sites of intrastrain recombination, homologous as well as site-specific. Recombinations occurred in both internal repeat sequences and regions of unique sequence. Some recombinations altered the open reading frames of known viral proteins and others duplicated sequence encoding the lytic origin of viral replication.

PCR amplification, cloning, and sequencing the EBNA-2 gene from several different specimens of HLP demonstrated the presence of multiple coinfecting strains in some specimens. Additionally, a variety of different partial-deletion recombinants of the EBNA-2 gene were described. In some cases, these recombinant viral variants apparently evolved within the lesion from a parental strain containing an intact EBNA-2 gene; whereas in other cases, the lesion appears to have been infected with a partial-deletion viral variant.

During productive replication in HLP, EBV undergoes frequent homologous and site-specific recombination within both unique and repeated sequences, generating viral variants. The consequences of these recombination events are not clear, but viral variants of EBV may possess altered biologic properties and possibly enhanced pathogenicity.