FUNCTIONAL ACTIVATION OF THE p53 TUMOR SUPPRESSOR IN NON-TUMORIGENIC VARIANTS OF THE HELA CERVICAL CARCINOMA CELL LINE

by

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Abstract

Two non-transformed revertants of the HeLa (ATCC CCL2) cell line have been isolated using a selection procedure based on prolonged retention of the fluorescent dye, rhodamine 123, within the mitochondria of carcinoma cells versus normal epithelial cells. Unlike the parental HeLa, the revertant cells exhibited a flat non-refractile morphology, failed to grow in suspension culture, had more than 100-fold lower cloning efficiencies in semi-solid medium, and failed to induce subcutaneous tumors when injected into nude mice. Both clones have retained a revertant phenotype after more than three years of continuous culture.

Somatic cell fusion experiments suggested that revertant cells had sustained a mutation or mutations that resulted in the activation of a dominant suppressor of transformation. Molecular characterization of these revertants demonstrated that the HPV-18 sequences present in the parental HeLa cells are retained in the revertants. Expression of the HPV-18 E6 and E7 at both the mRNA and protein levels were comparable to those seen in the parental HeLa line, suggesting that loss of the viral oncogenes was not responsible for loss of the tumorigenic phenotype in either clone. Among other possibilities, it was reasoned that a change in a regulator of the HPV oncoproteins or of the Rb and p53 genes could have interrupted the oncogene-suppressor association and have thus led to an activation of either Rb or p53. Using immunoaffinity purification and immunoblotting experiments no difference in Rb steady state levels were observed but increased p53 protein levels were found in both revertants as compared to Hela.. It is shown that functional activation of the p53 pathway is an obligatory step in the reversion process. The HeLa cells express wild-type p53 whose protein levels are maintained too low for its growth supressory functions to be manifested, due to the presence and function of the E6 protein. Increased steady state p53 levels in the revertants despite the presence of the E6 protein correlate precisely with the reversion of the transformed phenotype.

Ectopic expression of the wild-type p53 gene by retroviral integration was shown to decrease the clonigenicity if the Hela cell in soft agar by a hundred fold. The p53 gene in HeLa ATCC CCL2 has been previously characterized as wild-type. Mutation in the p53 cDNA's from HeLa and revertants has not been detected by RNAase protection assay or direct sequencing. Increased protein levels of p53 protein in the revertants as compared to HeLa were thus anticipated and found to result in a correspondingly differential manifestation of wild-type p53 function between the three cell lines. First, in the revertants as in non-transformed cells carrying the wild-type p53 gene, p53 protein is inducible upon treatment with Actinomycin D and γ–irradiation with kinetics of induction and induced p53 protein levels both being at least ten-fold higher than in Hela. Secondly, p53-specific transcriptional activity is detectable in the revertants. Specifically, transcription of the chloramphenicol acetylatransferase (CAT) gene driven from a promoter with multiple p53 consensus binding sites and a TATA box is detectable by CAT enzyme assay only in the revertants and not in HeLa. Treatment with Actinomycin D and γ–irradiation inreases the transactivating activity of endogenous p53 in the revertants thirty-fold. Moreover, downstream effectors of the transcriptionally active p53, namely the waf-1 and gadd-45 genes, are expressed in the revertants but not in HeLa. Expression of the gadd-45 mRNA is constitutive in the revertants while waf-1 mRNA is inducible by irradiation. p53-specific DNA binding activity in EMSA assays is also inducible by γ–irradiation and actinomycin D in the revertants and not in HeLa cells. Taken together, studies of the functional properties of endogenous p53 in revertants and HeLa correlate the transcriptional activity of p53 with the reversion from cervical carcinoma and offer valuable evidence that the transcriptional properties of the wild-type p53 contribute to its role in growth regulation.

An *in vitro* ubiquitination-degradation system was used to investigate the causes of increased p53 protein in the revertants. It was shown that differential targeting of the p53 protein for proteolysis occurs between HeLa and revertants since ubiquitination-dependent degradation of *in vitro* translated p53 protein is faster upon incubation with HeLa than with revertant cell lysate. This differential degradation of p53 in revertant cell extracts resulted from incomplete ubiquitination of the p53 protein. Protein-protein interaction studies in HeLa and revertants showed that p53-E6 binding in HeLa but not ine either revertant suggesting that the differential degradation of p53 in he revertants was caused by loss of E6 function. Furthermore p53 in HeLa and HA was associated with the cell cycle regulator p34cdc2, while in the HF cell line this

association was undetectable. p34cdc2 has been previously characterized as a modulator of p53 phosphorylation both *in vitro* and *in vivo* and phosphorylation of p53 at the p34cdc2 site has been found necessary for the ubiquitin-dependent degradation of the p53 protein. Thus, in the HF cell line, differential degradation of endogenous p53 may result from the failure of p53 to associate with the cell cycle regulator p34cdc2 and be phosphorylated by it.

Further characterization of the HeLa revertants included protein-protein interaction studies in the Rb pathway that showed differential binding of Rb to the transcription factor E2FI. Cell-cycle dependent binding and inhibition of this transcription factor is a known growth suppressory function of Rb. This result suggests that a common regulator of the p53 and Rb pathway may have been the target of the reversion event in this cell line. In order to exclude that reversion had resulted from E6 and/or E7 mutation we expressed ectopically by retroviral integration HPV-16 E6, E7 or a combination of the two and observed no effect on the revertant phenotype suggesting that the reversion event has bypassed the E6 and E7 functional pathways. Ectopic expression of SV40 Large T antigen, however, increased the clonigenicity in soft agar of the HA but not the HF cell line by -fold. Hence SV40 large T possesses a biochemical function that can override the cause of reversion in the HA cell line.

Taken together these results clearly establish that functional activation of p53 in two independent HeLa revertants is the result of change in two different pathways. They also show the great promise of the HeLa revertant system as a tool for the study of cervical transformation and for the involvement of the p53 pathway in this type of transformation. The process of identification of the molecular events that led to p53 and Rb activation in the revertants will provide valuable insight into the interplay of factors important for cell growth and cell growth suppression, and may identify potential targets for cancer therapy.

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INTRODUCTION

The HeLa transformant-revertant system

Non-transformed variants (revertants) have been isolated from a wide variety of oncogene transformed rodent cell lines after chemical mutagenesis (Zarbl et al., 1987, Haynes and Downing 1988), insertional mutagenesis (Kho and Zarbl, 1991) or after transfection with genomic DNA or cDNA expression libraries prepared from normal cells (Schaefer et al., 1988; Noda et al., 1989). Each of the two former techniques would have resulted in the loss of the transformed phenotype by causing a mutation either within the transforming oncogene itself or within a transformation effector gene whose products comprise and/or regulate the biochemical pathways of cell transformation (Boylan et al., 1991; Kho and Zarbl, 1991). A revertant phenotype could also arise from functional activation or ectopic expression of tumor suppressor genes. Thus the study of *in vitro* systems comprising of transformed cells and their non-tumorigenic variants culminates in identifying the gene or genes that have been the target of mutation during the reversion event. The ambition in producing such *in vitro* systems is to elucidate the mechanisms that lead to or prevent cellular transformation and tumorigenesis. Transformed cell/ revertant cell systems have been used succesfully to identify the *ras* suppressor *Krev* gene (Noda et al., 1989; Kitayama et al., 1989; Kitayama et al., 1990) and transformation effector genes (Boylan et al., 1991; Kho et al., 1991; Van Amsterdam et al., 1993).

A major difficulty of the transformed cell / revertant cell approach is the selection of a phenotypic characteristic that differentiates the few revertant cells that have arisen within the transformed cell population. Zarbl et al., (1987) have used loss of the prolonged rhodamine 123 dye retention phenotype to isolate revertants of v-fos-transformed Rat-1 fibroblasts by fluorescence-activated cell sorting (Zarbl et al., 1987). While prolonged retention of rhodamine 123 is limited to v-fos (Zarbl et al., 1987) and v-jun (our unpublished results) transformed rodent fibroblasts, this phenotype is common to almost all human carcinoma cell lines (Summerhayes et al., 1982). Thus the loss of prolonged rhodamine retention could be a universally acceptable procedure for the isolation of revertants of transformed human cells. We chose HeLa, a human epithelioid adenocarcinoma of the cervix, as the parental cell line from which to isolate revertants by selection for loss of prolonged rhodamine retention. Two stable revertant cell lines, HA and HF, have thus been isolated from the earliest available HeLa isolate, clone ATCC CCL2 following exposure to the mutagen ethylmethylsulfonate. The revertant cell lines have lost the ability to grow in suspension culture or in soft agar medium, as well as the ability to form tumors in nude mice. The transformed cell lines of the rodent systems that had been studied previous to our HeLa system were typically generated by the introduction of an activated oncogene such as *ras* or *fos* in an established cell line and therefore the genetic changes leading to transformation are partially defined. The HeLa cell, however, was a fully transformed cell derived from a

malignant, metastatic, lethal tumor. Cellular transformation is the hallmark of carcinogenesis and the phenotypic sum of multiple genetic abberations. In general, the extent of malignancy of a tumor is thought to directly correlate with the number of genetic abberations accumulated. Thus the genetic abberations in Hela would be far greatly complicated as compared to that of their rodent counterparts. To our advantage, however, as the first human cell line to have been propagated in cell culture, HeLa has been extensively characterized and used in numerous studies. More importantly, in recent years substantial research progress has shed light into the etiology of cervical carcinoma and has implicated biochemical pathways which are being actively investigated by numerous laboratories. In a way, what is known about cervical carcinoma could be considered the equivalent of knowing the identity of the transforming oncogene in the rodent systems, and thus justifies the selection of HeLa for the studies presented in this thesis.

Cervical Carcinoma and Human Papilloma Virus Transforming Viral Oncogenes

Cervical carcinoma, is the second most common gynaecological cancer. It is characterized by high metastatic potential and poor prognosis and claims about 500,000 lives world-wide every year. Epidemiological data have linked a sexually transmitted agent in the development of this type of cancer Between 85% and 95% of cervical carcinomas have been found to contain integrated viral genomic sequences of Human Papilloma Viruses -16, -18, -31, -33 and -39 the so called "high risk" HPV's (Durst et., 1983; zur Hausen, 1987 and 1991; Schiffman et al., 1991). In benign and premalignant lesions the HPV DNA exists episomally, while in malignant lesions and cervical carcinoma cell lines, HPV DNA is integrated, often in multiple copies, with preservation of the E6 and E7 open reading frames and disruption or deletion of the E1 and E2 genes (Schneider- Gadicke et al., 1988; Durst et al., 1987; Popescu et al., 1989; Lazo et al., 1989; Sousa et al., 1990). In some cases, which include the HeLa cell line, integration occurs in the vicinity of oncogenes - usually *c-myc* - and leads to their increased expression (Schneider- Gadicke et al., 1988). Chromosomal instability has been observed in HPV associated cancers with an increase from 33% to 82% in aneuploid cells in cervical lesion progressing from subclinical HPV infection through the stages of cervical intraepithelial neoplasia (Reid et al., 1984). The E6 and E7 genes of the "high risk" HPVs have been implicated as essential factors in cervical carcinogenesis because of their presence and expression in cervical carcinomas and carcinoma cell lines (Baker et al., 1987; El Awady et al., 1987; Schneider-Gadicke and Schwarz, 1986; Schwarz et al., 1985; Seedorf et al., 1987; Smotkin and Wettstein, 1986; Roggenbuck et al., 1991). Numerous studies have demonstrated the transforming properties

of the two viral oncogenes (reviewed in Mansur and Androphy, 1993). Most importantly, HPV-16 and -18 E6 and E7 can immortalize primary human keratinocytes (Kaur et al, 1988; Hawley-Nelson etal.. 1989; Munger et al., 1989; Hudson et al., 1990; Barbosa et al., 1991; Halbert et al., 1991; Schiller et al., 1989) and their maintained expression has been reported as both necessary and sufficient for tumorigenicity and full blown transformation (Munger et al. 1989, Bosch et al., 1990, Miyasaka et al., 1991). Furthermore, ectopic expression of HPV -16 and -18 E6 and E7 genes alone in primary human keratinocytes is sufficient to induce aneuploidy (Hawley-Nelson et al., 1989). Thus, current theory holds that non-productive HPV infection is an early event in carcinogenesis, that contributes to deregulation of growth as well as to the genomic instability responsible for further progression towards malignancy.

Human papilloma viruses belong to the family of small DNA viruses that express genes which can function as dominant oncogenes in biological systems of *in vitro* transformation, although it should be noted that human papilloma viruses are the only small DNA viruses to have been implicated in tumorigenesis in their natural host. By the time a link was established between human papilloma viruses and cervical carcinoma, significant progress had been achieved in understanding the biochemical and transforming properties of viral oncogenes of the small DNA viruses and particularly SV40 Large T and the Adenovirus E1A and E1B. Most of what we now understand about the biochemical and biological function of the E6 and E7 proteins has been investigated by analogy to SV40 Large T and Adenovirus E1A and E1B. Thus the transforming functions of the E6 and E7 are largely attributed to their respective binding and inactivation of the p53 and Rb tumor suppressor proteins (Werness et al., 1990; Munger et al., 1989; Dyson et al., 1989). Like SV40 large T and Adenovirus E1A E7 has been shown to inhibit Rb binding to E2F a transcription factor involved in control of cell proliferation (Nevins, 1991; Nevins, 1992 ; Morris et al., 1993 ; Hiebert et al., 1993; Hiebert et al., 1992; Bagchi et al., 1990; Pietenpol et al., 1990; Pietenpol et al., 1991; Sarnow et al., 1982). Like SV40 large T and Adenovirus E1B (Deppert et al., 1989; Deppert et al., 1989; Tack et al., 1989; Tack et al., 1992 ; Wang et al., 1989; Yew et al., 1992), E6 binds p53 but rather than increasing its half-life as its two former counterparts , E6 targets p53 for degradation through a novel ubiquitination pathway (Scheffner et al., 1992; Scheffner et al., 1992; Scheffner et al., 1993). Athough E6 and E7 function is not limited to p53 and Rb binding, these are the single properties known to differentiate the high risk HPVs from the low risk HPVs and are thus considered the crux of their transforming function. An important point suggested by experimentation on both the relative transforming (Halbert et al., 1992) and transactivation properties (Storey et al., 1990; Munger et al., 1991) of the low risk versus the high-risk HPVs is that their differences are related to dose rather than to a qualitative difference in function.

Both the E6 (Schiller et al., 1984; Grossman et al., 1989; Barbosa et al., 1989) and E7 (Miyasaka et al., 1991; Greenfield et al., 1991; McIntyre et al., 1993; Takami et al., 1992) proteins are nuclear phosphoproteins containing the Cys-x-x-Cys motif known as zinc finger necessary for protein-protein interactions -including dimerization- that characterize and regulate the activiy of many cellular transcription factors. Figure I.2 is a schematic representation of the E7 and E6 proteins and key features in their amino acid sequences.

Like adenovirus E1A, the E7 protein has also been shown to bind p107 (Dyson et al., 1992), an Rb-like protein that regulates members of the E2 family of transcription factors other than E2F-1 and acts at different stages in the cell cycle (Jones, et al., 1990). The region of p107 binding overlaps with but is not identical to the Rb binding domain. HPV-16 E7 has also been shown to bind cdk2 and cyclin A. Structural homology between E7 and E1A in the carboxy terminus pointed out a casein kinase II recognition site (Barbosa et al. 1990, Firzlaff et al., 1991) and E7 was found to be phosphorylated at serines 31 and 32 within this CKII site. High risk E7 is more highly phosphorylated than low risk E7 and a preference of Rb for the fully phosphorylated E7 may explain the differences in Rb binding between low risk and high risk E7 (Gage et al., 1990). Like Adenovirus E1A, E7 can transactivate the E2 promoter (Phelps et al., 1988; Phelps et al., 1987) apparently through release of E2F from its complex with Rb (Watanabe et al., 1990; Edmonds and Vousden, 1989; Schiller et al., 1990; Phelps et al., 1989; Phelps et al., 1992). Consequently the CKII site is important to the transactivation function of E7 (Storey et al., 1990; Watanabe et al., 1990; Edmonds and Vousden 1989; Schiller, J.T., Phelps et al., 1989). Again in an Rb-dependent manner (Banks et al., 1990) and much like E1A, E7 can induce DNA synthesis in quiescent rodent cells (Banks et al., 1990a; Banks et al., 1990b; Rawls et al., 1990). E7 is considered to be primarily responsible for the chromosomal instability associated with HPVs since its introduction in human keratinocytes was shown to produce cell lines with an extended life span and ploidy and chromosomal aberrations similar to those seen in cells transformed by the full length of the HPV genome (Hashida and Yasumoto, 1991). It is inknown however whether the genomic instability is related to the ability of E7 to induce DNA synthesis or an indirect result of of the E7- induced cell growth.

E6 is a strongly basic nuclear phosphoprotein with two putative zinc fingers. Thus, E6 has the characteristics of a DNA binding factor, although no specific sequence interactions have been reported reproducibly (Schiller et al., 1989; Gius et al., 1988; Imai et al., 1989). Since both the SV40 and Adenovirus viral oncogene products targeted Rb and p53, once it was shown that E7 bound Rb, E6 became the reasonable candidate to bind p53. In mixing experiments of *in vitro* translated E6 and p53, E6 was co-immunoprecipitated by an anti-p53 antibody (Scheffnner et al., 1990). It has been postulated that the first zinc-binding finger of HPV-16 E6 is necessary for targeting of p53 for degradation and the second is responcible for p53 binding (Crook et al.,

1991a). Binding of E6 to p53 occurs in a bimodal complex and is dependent upon the presence of a a 100kDa polypeptide, termed E6-AP (Scheffner et al., 1993) which has recently been cloned (Huibregtse et al.,) and found to function as a ubiquitin ligase (Scheffner et al., 1993).

Most of what we know about the function of E6 is related to its interaction with the p53 gene product. HPV-16 and -18 E6 have been immunoprecipitated from carcinoma cell lines with integrations of the respective HPV DNA (Androphy et al., 1987; Banks et al., 1987; Banks et al., 1987; Sedman et al., 1991; Androphy et al., 1985) as well as from HPV-immortalized keratinocytes (Hawley-Nelson et al., 1989; Sedman et al., 1991; Halbert et al., 1991) and in all cases its levels of expression appears were very low and "almost certainly much less than p53 in a cell" as quoted by Mansur and Androphy. Since E6 targets p53 for degradation without being itself degraded, even low levels are enough to lower p53 in the HPV containing cell lines to levels that although detectable, release the cell from the growth inhibitory effects of p53. By immunoblot the levels of p53 in HPV-16 E6 expressing keratinocytes do not always differ significantly from their primary cells of origin but the half-life of the p53 protein is always reduced (Band et al., 1990; Hubbert et al., 1992). Damage to DNA in both prokaryotic and eukaryotic cells leads to transient delays of cell cycle progression with blocks in the passage from the G1 phase to S and from the G2 phase to M (Tolmach et al., 1977; Painter and Young, 1980; Lau and Pardee, 1982; Weinert and Hartwell, 1988; Kaufmann et al., 1991; O 'Connor et al., 1992). Kastan et al., (1991) have implicated p53 in G1 arrest following γ-irradiation. Comparison between primary cells and cell lines transformed by HPV-16 DNA has shown that high risk E6 can interfere with this p53-mediated block in G1 upon DNA damage (Kessis et al., 1993). HPV-16 E6 and to a lesser extent HPV-6 E6 have also been shown to counteract the repressive effects of p53 on p53-repressible promoters in cotransfection assays (Lechner et al., 1992). The inhibition of p53-dependent repression was fully conferred by E6 mutants defective for p53 degradation but not for p53 binding. Conversely, HPV-18 and -16 E6 have been shown to transactivate basal promoters and mutations within the p53-binding zinc finger reduced E6 dependent transactivation (Sedman et al., 1991; Crook et al., 1991; Gius et al., 1988; Desaintes et al., 1992). However, mutations that abolished p53 binding *in vitro* remained transcriptionally competent in 3T3 cells suggesting that E6 transactivation may not be p53-dependent. Biochemical comparisons between the high risk and low-risk HPV E6 are less conclusive with respect to relevance to transformation than the same type of studies carried out for the E7 protein. While the Howley group has never been able to detect E6-p53 binding between low risk E6 and p53 (Scheffner et al., 1992) and have shown that only HPV-16 and -18 can target p53 for degradation in an ATP and ubiquitin dependent manner (Scheffner et al., 1990) they have later shown that HPV-6 -11 and -16 E7/E6 fusion proteins of which the E7 portion can bind an Rb peptide, all target the Rb peptide for degradation. The Vousden group has shown that HPV-6 E6

can bind p53 -albeit with lower affinity than HPV-16- but it cannot target p53 for degradation (Crook et al., 1991). Finally, the transactivation properties of the high and low risk HPV E6 appeared to be equally high (Sedman et al., 1991; Crook et al., 1991; Gius et al., 1988; Desaintes et al., 1992). Recently, two groups have reported that a number of other cellular factors can be bound by a glutathione column loaded with GST-E6 (). However, the identity of these proteins and the significance of their binding to E6 remains unknown.

Cervical carcinoma, Rb and p53

Rb and p53 are common targets of the viral oncogenes of the small DNA animal viruses (Kaelin et al., 1990; Ludlow et al., 1989; Schmieg et al, 1984). Full blown transformation can be conferred by these viruses to established cell lines only when both Rb and p53 are associated with a viral oncogene. The association is thought to inactivate the normal tumor suppressor functions of Rb and p53. Deregulation of both normal Rb and normal p53 function is a requirement in cervical carcinoma: all the cervical carcinoma cell lines tested express "high risk" HPV E6 and E7 or have sustained mutations in both Rb and p53 (Scheffner et al., 1991; Crook et al., 1992a; Crook et al,, 1992b; Crook et al., 1991).

The Rb tumor suppressor gene is a nuclear phosphoprotein with the biochemical properties of a transcription factor and the functions of a cellular growth regulator. Rb protein is expressed throughout the cell cycle and is a substrate for multiple phosphorylation which occurs progressively during the cell cycle (Firzlaff et al., 1989; Barbosa et al., 1990; Firzlaff et al., 1991, Taya et al., 1989; Thomas et al., 1991; Hinds et al., 1992; Dowdy et al., 1993; Yen et al., 1994; Zhang et al., 1994). Association between Rb and viral oncogenes prevents Rb binding to E2F a transcription factor involved in the control of cell proliferation (Sarnow et al., 1982; Bagchi et al., 1990; Pietenpol et al., 1990; Chellappan et al., 1991; Nevins et al., 1991; Pietenpol et al., 1991; Hiebert et al., 1992; Nevins, 1992; Nevins, 1992; Hiebert et al., 1993; Morris et al., 1993). Complex formation between Rb and E2F-1 prevents the latter from activating promoters of genes such as c-myc which are important signals for growth (Kley et al., 1992; Lechner et al., 1992). It was discovered that rather than simply inhibiting transcription Rb- E2F1 complex formation turns a positive regulatory element within the c-myc promoter into a negative one (). Rb phosphorylation modulates Rb-E2F binding throughout the cell cycle (Chellappan et al., 1991; Chittenden et al., 1991; Helin et al., 1992; Shirodkar et al., 1992) and is in general thought to play

a pivotal role in the regulation of Rb tumor suppressor functions (Mittnacht et al., 1991; Mittnacht et al., 1991; Cobrinik et al., 1992; Hatakeyama et al., 1994; Mittnacht et al., 1994). Viral oncogenes as well as E2FI bind the underphosphorylated form of Rb which predominates in the G1 phase of the cell cycle and during growth arrest. Inhibition of E2F-Rb complex formation by the viral oncogenes is thought to allow passage from the G1 phase to S, the phase of DNA synthesis. Most of the Rb literature refers to the underphosphorylated 105 kDa Rb species predominant in G1 as the active form of the Rb protein that inhibits the G1/S transition, as opposed to the inactive phosphorylated 110 kDa form. However, the continuation of Rb phosphorylation beyond the G1/ S phase suggests the possibility of multiple Rb functions regulated by various degrees of phosphorylation. Regulation of Rb phosphorylation is at least in part mediated through the paracrine growth factor TGFβ1 (Seto et al., 1992). TGFβ1 effects on cell growth vary according to cell type but in primary human keratinocytes, the system that has been most widely used for the study of E6 and E7, treatment with TGFβ1 leads to cessation of growth and a concurrent decrease in c-myc transcription (Deb et al., 1992). Alteration of the state of the Rb protein is thought to play a role in both the growth arrest and the down regulation of c-myc expression (Seto et al., 1992).

The p53 tumor suppressor gene is currently the most commonly mutated gene in human cancers, with seventy percent of all tumors and tumor cell lines examined carrying an altered p53 gene sequence. The p53 mutations are most commonly missence point mutations occuring within the conserved domains of the p53 protein and result either in loss of growth-suppressor or -most often- in gain of growth-promoting function (Bargonetti et al., 1991; Kern et al., 1991; Kern et al., 1991; Zambetti et al., 1991; Bargonetti et al., 1992; Bargonetti et al., 1992; Farmer et al., 1992; Zambetti et al., 1992; Zambetti et al., 1993; Olson et al., 1994). Several sites where mutations are detected more frequently have been characterized as mutation hot spots and include codons 175, 248, 273 and 281 (Hollstein et al., 1991; Vogelstein, 1990; Levine, 1991). Different tumor tissue types show different bias with respect to mutation site. For example mutation in codon 175 is common in colon carcinoma but never detected in lung. Germ-line p53 mutations have also been detected in families with high cancer incidence in multiple tissues, a condition known as the Li-Fraumeni syndrome. The germ cells of these Li-Fraumeni patients are heterozygous for mutant and wild-type p53 while their tumors are homozygous for mutant p53 (Malkin et al., 1990 and 1992; Srivastana et al., 1990 and 1992; Togutsida et al., 1992; Iavarone et al., 1992; Li and Fraumeni 1969). Elevated wild-type p53 expression supresses the transformed phenotype of colon carcinoma, osteosarcoma and breast carcinoma cell lines (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Chen et al. 1991; Cheng et al., 1992; Wang et al., 1993). The exact mechanism or mechanisms through which wild-type p53 functions in

tumor/growth supression is not known, although p53 involvement in response to DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992; Kessis et al., 1993; Lowe et al., 1993; Lowe et al., 1994; Slebos et al., 1994), cell cycle control (Kastan et al., 1991; Perry et al., 1993; Slichenmyer et al., 1993; Chen et al., 1994; Slebos et al., 1994), transcriptional activation (Kern et al., 1991; Kern et al., 1991; Zambetti et al., 1991; Kern et al., 1992; Zambetti et al., 1992; Zambetti et al., 1993), transcriptional repression (Ginsberg et al., 1991; Santhanam et al., 1991; Subler et al., 1992; Kley et al., 1992; Chin et al, 1992; Lechner et al., 1992; Seto et al., 1992) and DNA replication (Wang et al., 1989; Friedman et al., 1990; Bargonetti et al., 1991; Bargonetti et al., 1992; Friedman et al., 1993; Reynisdottir et al., 1993; Wang et al., 1993; Moses et al., 1994) have all been implicated. An ongoing hypothesis which explains early involvement of p53 in carcinogenesis views p53 function as a safeguard of the integrity of the genome. Thus, when p53 function is normal, cells that cannot replicate properly -due either to lack of metabolites or to extensive DNA damage- stop dividing or die. However, when p53 function is perturbed offended cells can suffer further oncogenic changes and develop into cancer cells. p53-specific transcriptional activation of genes controlling the cell cycle, specifically the cyclin inhibitor cip-1/waf-1 (el Deiry et al., 1993) provides a direct link between p53 and the control of cell proliferation. Transcriptional activation and DNA binding are specific to wild-type p53 (Kern et al., 1991; Kern et al., 1991; Kern et al., 1992) as is the induction of the cyclin kinase inhibitor cip-1/waf-1 after γ-irradiation (Scott Lowe, personal communication). Thus it is thought that the transcription factor properties of wild-type p53 are essential for its role in cell growth control.

p53 is a 393 amino acid nuclear phosphoprotein whose structure resembles a transcription factor (Mathlasewski et al., 1984; Pennica et al., 1984; Soussi et al., 1987). The amino- terminus is highly acidic with a net charge similar to Herpes Simplex virus VP16 and yeast GAL-4. The carboxy- terminus is strongly basic as would be expected from a DNA binding domain and contains three nuclear localization signals (NLS) (Shaulsky et al., 1990). The predominant NLS is located adjacent to serine 315 (312 for mouse and rat p53) which is phosphorylated in vivo and in vitro by cdc2p34 (Milner et al. 1990; Sturzbecher et al., 1990; Bischoff et al., 1990). Thus phosphorylation of this serine residue could influence p53 subcellular localization and biological activity in a cell cycle dependent manner. Phosphorylation has also been speculated to be the mechanism through which p53 is stabilized in response to DNA damage or cellular stress. Lin and Desiderio have shown that phosphorylation of Serine 315 inhibits the ubiquitin dependent degradation of p53. The p53 has also been documented to be a substrate for casein kinase II () and the DNA () polymerase associated kinase.

The major mechanism of control of p53 expression appears to be through protein stability (post-transcriptional modification; Oren et al 1981, Reich et al. 1981). The hypothesis that HPV E6 targets p53 for ubiquitination was formulated when HPV-16 and -18 were found to

bind E6 *in vitro* and cervical carcinoma cell lines with HPV integrations that express the wild-type p53 mRNA but show low levels of p53 protein . HPV -16 and -18 E6 were consequently found to induce ATP and ubiquitin-dependent degradation of p53 in rabbit reticulocyte lysates (Scheffner et al.,1990) and a novel ubiquitin-dependent pathway has been implicated in p53 degradation (Scheffner et al., 1992a; Scheffner et al., 1992b; Scheffner et al., 1993). Ubiquitin-dependent degradation of p53 *in vitro* has been more recently shown to take place in the absence of E6 (Ciechanover et al., 1994). Wild-type p53 is a relatively short-lived protein with a half-life of about 3 hours in normal cells and twenty minutes in transformed cells. Endogenous levels of p53 protein are low in certain cell types and vary two- to four-fold between various stages of the cell cycle in p53 expressing cells. It is therefore possible that ubiquitin-dependent proteolysis is involved in the regulation of p53 protein expression in non-transformed cells and that E6 analogs may exist in such cells.

p53 was ound to transactivate the muscle creatine kinase (MCK) gene (Weintraub et al., 1993) and a p53 binding site was defined within the MCK promoter. p53 was subsequently shown to bind DNA both specifically (Kern et al., 1991b; Bargonetti et al., 1991) and non-specifically (Steinmeyer et al., 1988; Kern et al., *b*, 1991). When the p53 response element was included in basal promoters and placed 5' of the CAT reporter gene, the reporter was transactivated when co-transfected with wild-type p53 (Zambetti et al..,1992; Kern et al., 1992; Farmer et al., 1992). Furthermore when mutant and wild-type p53 where co-expressed there was a dominant effect of the mutant p53 that was related to decreased binding of presumed wild-type mutant oligomers to DNA (Kern et al. 1992). A number of promoters like those of *c-fos* and *c-jun* have now been shown to be repressed by p53. Repression does not occur though a p53 binding site and the promoters repressed by p53 are seemingly unrelated, indicating that a more general mechanism for suppression may be operative (Ginsberg et al., 1991; Santhanam et al., 1991; Subler et al., 1992; Kley et al., 1992; Chin et al, 1992; Lechner et al., 1992). Wild-type p53 transfected into p53 null cell lines was shown to supress a minimal promoter including only the adenovirus major late TATA element and the terminal deoxynucleotidyl transferase initiator. p53 was subsequently found to be able to complex with the TATA binding protein (TBP) and has been hypothesized to exert its supressive effects through the core transcriptional machinery (Seto et al., 1992).

Damage to DNA in both prokaryotic and eukaryotic cells lead to transient delays of cell cycle progression with blocks in the passage from the G1 phase to S and from the G2 phase to M. These blocks are presummed to prevent replication of damaged DNA and segregation of damaged chromosomes, respectively. p53 protein has been shown to be inducible in mouse fibroblast lines by treatment with ultraviolet light or UV-mimetic drugs due in large part to increased stability of the protein. The induction has also been shown to take place in vivo in human skin subjected to

UV. Similar responses have been shown after treatment with γ-irradiation or Actinomycin D (Kastan et al., 1991). Exposure to the inducing agents caused a temporary arrest in the G1 of the cell cycle in cells expressing wild-type p53 but not in cells with mutant p53. Furthermore, malignant cells lacking endogenous p53 partially restored the ability to arrest in G1 after γ-irradiation when they were transfected with a construct expressing wild-type p53, while overexpression of a mutant p53 in tumor cells with a wild-type endogenous p53 abbrogated the G1 arrest (Kuebritz et al., 1992). Cells derived from patients suffering with Ataxia Telangiectasia -a disease characterized by hypersensitivity to ionizing radiation, radioresistant DNA synthesis and a markedly increased incidence of cancer- failed to induce p53 and failed to arrest in G1, much in a manner similar to p53-deficient cells (Kastan et al., 1992). These results suggested a role for p53 in cell cycle control that requires the uninterrupted function of p53 inducing pathways. In the Ataxia Telangiectasia cells failure to induce p53 protein following γ-irradiation correlated with failure to induce the mRNA of the growth arrest and DNA damage inducible gene gadd-45, which may be involved in DNA repair. The lack of gadd45-mRNA induction, further correlated with lack of p53 binding to a site within intron 3 of the gadd45 gene. Zhan et al. 1993 provided further evidence suggesting that p53 involvement in gadd45 induction is specific to γ-irradiation and not other treatments. Thus DNA binding and transactivational properties of p53 were implicated in the response to DNA damage.

But not until the discovery of the cip1/waf1 gene had there been a link between the transcriptional properties of wild-type p53 and cell cycle control. The WAF-1 gene has been identified by means of its differential induction by wild-type but not mutant p53. The induction is thought to be mediated by direct transactivation of the waf-1 promoter since the identified p53 binding sites within this promoter can confer p53 specificity to reporter constructs (El Deiry et al., 1993). WAF-1 was then found to be identical to a gene called CIP-1 which encoded an inhibitor of cyclin/cyclin kinase complexes (Harper et al., 1993). Evidence has since been accumulating to verify the hypothesis that induction of p53 upon conditions of cellular stress or DNA damage leads to inhibitiion of cell cycle progression through the waf-1/cip-1 protein. All current evidence suggests that wild-type p53 function is absolutely essential to waf-1 induction (El Deiry et al., 1993).

Previous work has shown that p53 is functional in tetrameric complexes (Friedman et al., 1993). It appears that during the progression of the cell cycle, the wild-type p53 protein can assume two conformations, each of which may play a distinct role in the physiological life of the cell (Ullrich, et al., 1992). One conformation is specific to the wild-type protein and has been associated with the ability to block cell cycle progression. The other conformation seems to function in promoting cell proliferation and had originally been distinguishing mutant p53 from wild-type (Harlow et al., 1981; Yewdell et al., 1986; Gannon et al. 1990, Cook and Milner, 1990).

Milner and coworkers have demonstrated that when mutant and wild-type p53 are co-expressed *in vitro* (1:1 molecular ratio) complex formation between wild-type p53 and mutant p53 forced the wild-type p53 into the mutant conformation. Furthermore, Kern et al. showed that transiently transfected 175 mutant could reduce the p53 transactivation of reporter constructs in the presence of cotransfected wild-type p53. The work of Ullrich et al., (1992) showed that the allosteric effect of mutant p53 may not occur *in vivo* in the same direct manner as *in vitro.* In cells expressing endogenous mutant p53 and an inducible wild-type p53 gene both wild-type/wild-type p53 and wild-type/mutant complexes exist. In these cells the wild-type p53 can exert its antiproliferative effect and upon G1 arrest no wild type p53 is seen complexed with mutant. Finally in Saos-2 cells, which lack endogenous p53, simultaneous retroviral transfer and expression of the wild-type and a dominant mutant p53 genes was able to suppress anchorage independent growth and tumorigenicity much as expression of wild-type alone. Thus, in the *in vivo* contexts examined, wild-type p53 has a dominant effect over the mutants biochemically defined as dominant.

Cervical transformation and the HeLa/revertant system

The HeLa cells harbor approximately 10-20 copies of HPV-18 DNA per genome equivalent (Lazo et al., 1987; Lazo et al., 1989; Schwarz et al., 1985) and they express the E7 and E6 viral oncogenes. Continued expression of the E6/E7 genes has been found necessary for the maintenance of the transformed phenotype of the HeLa cell (Bosch et al., 1990; Bosch et al., 1991). The Rb protein was reported to be normal-sized in both its phosphorylated and non-phosphorylated forms in HeLa (Scheffner et al., 1991). p53 mRNA expression was not inhibited but the levels of the p53 protein were found to be undetectable (Matlashewski et al., 1986) or very low while p53 cDNA sequence was wild-type (Scheffner et al., 1991). p53 protein synthesis was, however, shown to take place when the endogenous p53 protein present was stabilized by transfection with a mouse mutant p53 and detected by immunoprecipitation (May et al., 1991). The latter result suggested that p53 is being rapidly degraded in the HeLa cell, presumably via the E6-mediated ubiquitination pathway.

This Research Thesis describes the initial characterization of non-transformed revertants isolated from HeLa cells on the basis of prolonged rhodamine 123. Initial phenotypic characterization of the revertants demonstrated that these cells lost most of the *in vitro* properties associated with transformed cells, including a transformed morphology, clonigenicity in semi-solid medium, and growth in suspension culture (Boylan et al, 1996). Stanbridge and co-workers

have previously carried out somatic cell hybridization experiments using HeLa cells and primary human fibroblasts and showed that the tumorigenicity of HeLa cells is suppressible (). They have futher demonstrated that a tumor suppressor gene harbored on the short arm of human chromosome 11, is able to suppress the tumorigenicity of HeLa cells (Maniatis et al., 1982; Srivatsan et al, 1986 ; Stanbridge et al., 1986). However, all of the non-tumorigenic cell hybrids generated from the fusion of HeLa cells and normal human fibroblasts invariably maintained the *in vitro* characteristics of the transformed parental Hela. Thus, loss of the tumor suppressor gene on chromosome 11 is likely to be associated with tumor progression rather than initiation of carcinogenesis. In contrast, the events that led to reversion in our system may have affected early pathways of transformation, such as E6 and E7 functional pathways. In rodent transformed cell / revertant cell systems reversion is often the cause of inactivatng mutation within the transforming oncogene (). Our knowledge of the involvement of E6 and E7 in cervical transformation makes each of these genes the analogue of the transforming oncogene in the rodent systems. It was therefore necessary to examine the possibility of functional inactivation of each of the two oncogenes.

Continued expression of the E6/E7 genes -which has been found necessary for the maintenance of the transformed phenotype of the HeLa cell (Schwarz et al., 1985; Schneider-Gadicke et al., 1986; Schneider-Gadicke et al., 1987; Bosch et al., 1990; Bosch et al., 1991)- was verified in the revertants. Retroviral transduction of HPV-16 E6, E7 or a combination of the two had no effect on the revertant phenotype. On the other hand, cell fusion experiments between HeLa and revertants raised the possibility that a tumor suppressor gene may have been activated during the reversion process. Since the transforming functions of the "high risk" HPV E6 and E7 are thought to result from their respective binding and inactivation of the p53 and Rb tumor suppressor proteins (Dyson et al., 1989; Munger et al., 1989; Munger et al., 1989; Werness et al., 1990) functional inactivation of the E6 and/or E7 could be manifested as activation of either Rb or p53 tumor suppressors. Such activation could conceivably be caused by the interruption of the association between the viral oncoprotein and cellular tumor suppressor. Disruption of the suppressor/inactivatingoncogene association could in turn result from a change in a regulator of the tumor suppressor or of the relevant HPV oncogene protein. We have thus examined the state of the p53 and Rb tumor suppressor genes in HeLa and revertant cells.

We found increased steady state levels of wild-type p53 in both revertants despite the continued presence of the E6 protein. Detectable Rb-E2FI binding in the revertants suggested functional Rb activation and E7 inactivation, particularly in tthe HF revertant. In addition, differences in protein-protein interactions in both the Rb and p53 pathways were observed between HeLa and the revertants. To this point our data had established that reversion of transformation was the result of functional inactivation of the E6 and E7 pathways in the

revertants. Increase of the levels of p53 was the most consistent and striking difference between HeLa and the revertants and the remainder of the research presented in this report focused on the study of the p53 gene product in our system. We have thus shown that wild-type p53 can suppress the HeLa phenotype. We have further succeeded in establishing that revertant p53 is wild-type and that wild-type-p53-specific functions are manifested in the revertants but not in HeLa. Finally, we have shown that decreased p53 proteolysis is the probable cause of increased p53 levels in the revertants with respect to HeLa. Loss of E6 function and of ubiquitin dependent degradation have been identified as the cause for functional p53 activation and a testable hypothesis has been formulated about the involvement of p34cdc2 in the functional pathway of the E6 viral oncogene. Although the extent of p53 contribution to the revertant phenotype remains to be assessed, the research presented here emphasizes the central role of the HPV and p53 pathways in cervical transformation and offers *in vivo* evidence for the link between the transcriptional activity of p53 and the control of cell growth. Most importantly, this work suggests that interference with the HPV functional pathway or activation of the p53 pathway may help control cervical carcinoma cell growth and may be reasonable approaches to the development of new therapeutic strategies.

CHAPTER ONE

Activation of tumor suppressor genes in two independent non-tumorigenic variants (revertants) of the HeLa cervical carcinoma cell line.

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[4] This author contributed the initial isolation and characterization of the revertant cell lines.

Running Title: Tumor suppressors in non-tumorigenic revertants of HeLa cells

Abstract

We report the isolation of two non-transformed revertants of the HeLa (ATCC CCL2) cell line. Revertant cells exhibited a flat non-refractile morphology, failed to grow in suspension culture, had more than 100-fold lower cloning efficiencies in semi-solid medium and failed to induce subcutaneous tumors when injected into nude mice. Somatic cell fusion experiments suggested that revertant cells had sustained a mutation or mutations that resulted in the activation of a suppressor of transformation. Molecular characterization of these revertants demonstrated that the HPV-18 sequences present in the parental HeLa cells are retained in the revertants. Expression of the HPV-18 E6 and E7 at both the mRNA and protein levels were comparable to those seen in the parental HeLa line. Retroviral transduction of HPV-16 E6, E7or a combination of the two had no effect on the revertant phenotype, suggesting that loss of the viral oncogenes was not responsible for loss of the tumorigenic phenotype in either clone. We have investigated whether the Rb and p53 genes have been reactivated during the reversion process. We found that increased steady state levels of wild-type p53 in the revertants despite the presence of the E6 protein correlate precisely with the reversion of the transformed phenotype. Differences in protein-protein interactions in the pathway of p53 and/or Rb among the three cell lines and the fact that retroviral transduction of the SV40 large T antigen increased clonigenicity in soft agar HA cell line only by a hundred-fold of the indicate that reversion in the two independent revertant clones was the result of two distinct events. Rb-E2FI binding in the revertants and particularly HF suggested functional Rb activation in this clone. Taken together these results clearly establish that the reversion of transformation in two independent HeLa daughter clones occured via two distinct mechanisms that result in functional inactivation of the E6 and/or E7 pathways.

Introduction

Non-transformed variants (revertants) have been isolated from a wide variety of oncogene transformed rodent cell lines after chemical mutagenesis (Zarbl et al., 1987), insertional mutagenesis (Kho et al., 1991) or after transfection with genomic DNA or cDNA expression libraries prepared from normal cells (Schaefer et al., 1988; Noda et al., 1989). Loss of the transformed phenotype can result from inactivation of the transforming oncogene or transformation effector genes, whose products comprise and/or regulate the biochemical pathways of cell transformation (Boylan et al., 1991; Kho et al., 1991). A revertant phenotype can also arise from activation or ectopic expression of tumor suppressor genes.

We have previously used loss of the prolonged rhodamine 123 dye retention phenotype to isolate revertants of v-fos-transformed Rat-1 fibroblasts using fluorescence-activated cell sorting (Zarbl et al., 1987). While prolonged retention of rhodamine 123 is limited to v-fos (Zarbl et al., 1987) and v-jun (our unpublished results) transformed rodent fibroblasts, this phenotype is common to almost all human carcinoma cell lines (Summerhayes et al., 1982). We were interested in studying revertants of a human tumor cell line and chose as our parental cell line, HeLa, an epithelioid adenocarcinoma of the cervix. Two stable revertant cell lines, HA and HF, have been isolated from HeLa (ATCC CCL2) following exposure to the mutagen ethylmethylsulfonate. The revertant cell lines were selected for their non-transformed morphology and for loss of prolonged rhodamine retention by fluorescence-activated cell sorting; they have lost the ability to grow in suspension culture or in soft agar medium, as well as the ability to form tumors in nude mice. The studies presented in this paper were aimed at identifying molecular events that may be central to the mechanism of transformation of HeLa and may thus be causative factors of cervical carcinoma.

Cervical carcinoma, the second most common gynecological cancer is characterized by high metastatic potential and poor prognosis and claims about 500,000 lives world-wide every year. Epidemiological data have linked a sexually transmitted agent in the development of this type of cancer: as many as 90% of cervical carcinoma has been found to contain integrated viral genomic sequences of Human Papilloma Viruses -16, -18, -31, -33 and -39 the so called "high risk" HPV's (Durst et al., 1983; zur Hausen et al., 1987; zur Hausen et al., 1991; Schiffman et al., 1991). In benign and premalignant lesions the HPV DNA exists episomally, while in malignant lesions and cervical carcinoma cell lines HPV DNA is integrated, often in multiple copies, with preservation of the E6 and E7 open reading frames and disruption or deletion of the E1 and E2 genes (Schneider- Gadicke et al., 1988; Durst et al., 1987; Popescu et al., 1989; Lazo et al., 1989; Sousa et al., 1990). In some cases, which include the HeLa cell line, integration occurs in the vicinity of proto-oncogenes - usually *c-myc* - and leads to increased expression of this gene

(Schneider- Gadicke et al., 1988). The E6 and E7 genes of the "high risk" HPVs have been implicated as essential factors in cervical carcinogenesis by virtue of their presence and expression in cervical carcinomas and carcinoma cell lines (Baker et al., 1987, El Awady et al., 1987, Schneider-Gadicke and Schwartz, 1986, Schwarz et al, 1985, Seedorf et all 1987, Smotkin and Wettstein, 1986, Roggenbuck et al. 1991). Numerous studies have demonstrated the transforming properties of the two proteins (reviewed in Mansur and Androphy, 1993). Most importantly , HPV-16 and -18 E6 and E7 can immortalize primary human keratinocytes (Kaur et al, 1988; Hawley-Nelson etal.. 1989; Munger et al., 1989; Hudson et al., 1990; Barbosa et al., 1991; Halbert et al., 1991; Schiller et al., 1989) and their maintained expression has been reported as both necessary and sufficient for tumorigenicity and full blown transformation (Munger et al. 1989, Bosch et al., 1990, Miyasaka et al., 1991). The transforming functions of the E6 and E7 are thought to result from their respective binding and inactivation of the p53 and Rb tumor suppressor proteins (Wernesset al., 1990 ; Munger et al., 1989; Dyson et al., 1989). Rb and p53 are common targets of the viral oncogenes of the small DNA animal viruses (Kaelin et al., 1990; Ludlow et al., 1989; Schmieg,et al., 1984). Full blown transformation can be conferred by these viruses to established cell lines only when both Rb and p53 are associated with a viral oncogene. The associations with viral oncogenes are thought to inactivate the normal tumor suppressor functions of Rb and p53. Specifically, association of Rb with SV40 large T, Adenovirus E1A and E7 has been shown to inhibit Rb binding to E2F a transcription factor involved in control of cell proliferation (Nevins, 1991; Nevins, 1992; Morris et al., 1993; Hiebert et al., 1993; Hiebert et al., 1992; Bagchi et al., 1990; Pietenpol et al., 1990; Pietenpol et al., 1991; Sarnow et al., 1982). Association of SV40 large T and Adenovirus E1B with p53 binds and inactivates p53 (Deppert et al., 1989; Deppert et al., 1989; Tack et al., 1989; Tack et al., 1992; Wang et al., 1989; Yew et al., 1992) but results in p53 stabilization, while HPV E6 is thought to inactivate p53 by targeting it for degradation through a novel ubiquitination pathway (Scheffner et al., 1992; Scheffner et al., 1992; Scheffner et al., 1993). Deregulation of normal Rb and p53 function appears to be a requirement in the pathogenesis of cervical carcinoma: all the cervical carcinoma cell lines tested express "high risk" HPV E6 and E7 or have sustained mutations in both Rb and p53 (Scheffner et al., 1991; Crook et al., 1992; Crook et al., 1992; Crook et al., 1991).

The Rb tumor suppressor gene is a common target of DNA viral oncogenes namely the SV40 large T, adenovirus E1A and "high risk"-HPV E7. Rb tumor suppressor functions are abbrogated (Schmieg et al., 1984; Ludlow et al., 1989; Munger et al., 1989; Kaelin et al., 1990). Rb protein is a substrate for multiple phosphorylation which occurs progressively during the cell cycle (Firzlaff et al., 1989; Barbosa et al., 1990; Firzlaff et al., 1991, Taya et al., 1989; Thomas et al., 1991; Hinds et al., 1992; Dowdy et al., 1993; Yen et al., 1994; Zhang et al., 1994). Association between Rb and viral oncogenes prevents Rb binding to E2F, a transcription factor

involved in control of cell proliferation (Sarnow et al., 1982; Bagchi et al., 1990; Pietenpol et al., 1990; Chellappan et al., 1991; Nevins et al., 1991; Pietenpol et al., 1991; Hiebert et al., 1992; Nevins, 1992; Nevins, 1992; Hiebert, 1993; Morris et al., 1993). Rb phosphorylation modulates Rb-E2F binding throughout the cell cycle (Chellappan et al., 1991; Chittenden et al., 1991; Helin et al., 1992; Shirodkar et al., 1992) and is generally thought to play a pivotal role in the regulation of Rb tumor suppressor functions (Mittnacht et al., 1991; Mittnacht et al., 1991; Cobrinik et al., 1992; Hatakeyama et al., 1994; Mittnacht et al., 1994). Viral oncogenes as well as E2FI bind the underphosphorylated form of Rb which predominates in the G1 phase of the cell cycle. Inhibition of E2F-Rb complex formation by the viral oncogenes is thought to allow passage from the G1 phase to S, the phase of DNA synthesis.

The p53 tumor suppressor gene is currently the most commonly mutated gene in human cancers, with seventy percent of all tumors and tumor cell lines examined carrying an altered p53 gene sequence. Elevated wild-type p53 expression supresses the transformed phenotype of colon carcinoma, osteosarcoma and breast carcinoma cell lines (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Chen et al. 1991; Cheng et al., 1992; Wang et al., 1993). The exact mechanism or mechanisms through which wild-type p53 functions in tumor/growth supression is not known, although the involvement of p53 in response to DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992; Kessis et al., 1993; Lowe et al., 1993; Lowe et al., 1994; Slebos et al., 1994), cell cycle control (Kastan et al., 1991; Perry et al., 1993; Slichenmyer et al., 1993; Chen et al., 1994; Slebos et al., 1994), transcriptional activation (Kern et al., 1991; Kern et al., 1991; Zambetti et al., 1991; Kern et al., 1992; Zambetti et al., 1992; Zambetti et al., 1993) and DNA replication (Wang et al., 1989; Friedman et al., 1990; Bargonetti et al., 1991; Bargonetti et al., 1992; Friedman et al., 1993; Reynisdottir et al., 1993; Wang et al., 1993; Moses et al., 1994) have all been demonstrated. An ongoing hypothesis which explains early involvement of p53 in carcinogenesis views p53 function as a guardian of the genome integrity. Thus, when cells with normal p53 cannot replicate properly due either to lack of metabolites or to extensive DNA damage, p53 invokes the cells to stop dividing or die. When p53 function is perturbed however, offended cells can continue to divide and to accumulate further oncogenic changes that allow progression towards cancerous growth. p53-specific transcriptional activation of genes controlling the cell cycle, specifically the cyclin inhibitor cip-1/waf-1 (el Deiry et al., 1993) provides a direct link between p53 and the control of cell proliferation. Transcriptional activation and DNA binding are specific to wild-type p53 (Kern et al., 1991; Kern et al., 1991; Kern et al., 1992) as is the induction of cip-1/waf-1 after γ-irradiation (El Deiry et al., 1993). Thus, it is thought that the transcription factor properties of wild-type p53 are essential for its role in cell growth control.

The major mechanism for control of p53 expression in cells appears to be through protein stability (post-transcriptional modification; Oren et al 1981, Reich et al. 1981). E6 proteins HPV-16 and -18 bind p53 in vitro and cervical carcinoma cell lines with HPV integrations carry the wild-type p53 gene, although the cervical carcinoma cells express the p53 mRNA, but they show low levels of p53 protein, leading to the hypothesis that HPV E6 targets p53 for ubiquitination consistent with this hypothesis. HPV -16 and -18 E6 were thus found to induce ATP and ubiquitin-dependent degradation of p53 in rabbit reticulocyte lysates (Scheffner et al. ,1990), and a novel ubiquitin-dependent pathway has been implicated in p53 degradation (Scheffneret al., 1992; Scheffner et al., 1992; Scheffner et al., 1993). Furthermore, there is evidence that decreasing p53 is essential for transformed phenotypes.

The HeLa cells harbor approximately 10-20 copies of HPV-18 DNA per genome equivalent (Lazo et al., 1987; Lazo et al., 1989; Schwarz et al., 1985) and they express the E7 and E6 viral oncogenes. The Rb protein was reported to be normal-sized in both its phosphorylated and non-phosphorylated forms in HeLa (Scheffner et al., 1991). p53 mRNA expression was not inhibited in HeLa cells, but the levels of the p53 protein were found to be undetectable (Matlashewski et al., 1986) or very low and p53 cDNA sequence was wild-type (Scheffner et al., 1991). p53 protein synthesis was shown be normal when p53 protein could be stabilized by transfection of HeLa cells with a mutant p53 (May et al., 1991), suggesting that wild-type p53 rapidly degraded in the HeLa cell, presumably via the E6-mediated ubiquitination pathway.

We have investigated whether inactivation of viral oncogene function has contributed to the non-transformed phenotype of the HA and HF revertants. We have identified no differences in the expression of the E6 and E7 genes between HeLa and revertants but found changes that suggested functional activation of the Rb and p53 patways in the revertants. Our results established that the reversion from transformation in the two independent HeLa revertants was the result of two distinct events and suggested inactivation of the E6 and/or E7 pathway with the consequent activation of the p53 and Rb pathways. Identification of the molecular events that led to p53 and Rb activation in the revertants will provide valuable insight into the interplay of factors important for cell growth and cell growth suppression, and may identify potential targets for cancer therapy.

Materials and Methods

Cell Culture

HeLa CCL2, C4I, C4II, FS4 and Saos-2 cells were purchased from American Type Culture Collection. A431 cells were kindly provided by Robert Weinberg's Laboratory. C4I and C4II cells were grown in Waymouth's Medium supplemented with 10% fetal bovine serum (FBS). FS4, A431, and Saos-2 cells were grown in DMEM with 10% FBS. HeLa and revertants were grown in MEMN + 10% FBS or in MEMN +10% NuSerum (Collaborative Research). Growth in soft agar was assessed by seeding 1 X 10^2, 1 X10^3 and 1 X 10^4 cells in 4 ml of MEMN containing 10% fetal bovine serum and 0.3% molten Difco noble agar. Plates were kept at 4° C for 15 min before incubation at 37° C in a 5% CO_2 atmosphere. Plates were fed with 2.0 ml of 0.3% agar medium every 7-10 days.

Growth in suspension cultures was assessed by seeding approximately 1X 10^6 cells into 100 ml of MEMN +10% FBS in a 250 ml spinner flask. The flasks were incubated at 37° C in a 5% CO_2 atmosphere with continuous stirring. Two flasks of each cell type were grown in parallel and two 1ml aliquots were removed from each flask every two days and cell numbers were determined using a Coulter Counter (Coulter Electronics).

Fluorescence-activated cell sorting and isolation of revertants

Cells were stained by exposure to 10 μg/ml of rhodamine 123 (Sigma) in complete culture medium at 37° C for 30 min. Cells were then washed 4 times with complete medium, each time for 30 min at 37° C and were then incubated for an additional 6 to 8 h before preparation for sorting or analysis. To increase the frequency of revertants, approximately 1 X10^7cells were treated with ethane methylsulfonate at a concentration of 3 mM for 18h. The cells were then allowed to recover for 4 days in normal media before they were harvested by trypsinization, suspended in MEMN +10% fetal bovine serum, and filtered through 70 μm nylon mesh. Cells were then stained with rhodamine 123 and the relative fluorescence intensity (rhodamine 123 retention) and relative size (forward angle light scatter) were measured with a FACSTAR^{plus} fluorescence-activated cell sorter (Becton-Dickinson). Viable cells that did not retain rhodamine 123 were collected using previously described parameters (Zarbl et al., 1987). Colonies visible after 7 days were again stained with rhodamine 123 and examined directly by epifluorescent illumination using an inverted microscope. Colonies that failed to retain the dye were isolated using cloning cylinders and purified by repeated cloning.

Tumorigenicity Assay

The tumorigenic potential of the cell lines was evaluated by injection into athymic (nude) mice (Charles River). Cells were harvested by trypsinization, washed and suspended in 0.5-1.0 ml of MEMN. Subcutaneous injections of 1 X 10^7 cells were done with a 23 gauge needle over the right rear haunch of weanling animals. Both male and female animals were injected. The sites of injection were palpated on a weekly basis for the appearance of tumors.

Fusion Hybrid formation and soft agar growth:

HeLa, HF and HA cells were grown to 80% confluence in MEMN+10% Nu Serum. 10^7 cells of each cell line were harvested by trypsinization electroporated with 1μg of either PY3 (HeLa) or pMEXneo (revertants) using a GenePulser apparatus (BioRad). Stable transfectants of HeLa were then selected in media containing hygromycin at a concentration of 200μg/ml and stable transfectants of revertants were selected in media containing G418 at a concentration of 200μg/ml. Clones HeLa-PY3-9, HFneo10 and HAneo17 were characterized for alkaline phopshatase activity and used for fusion experiments. The cells from one plate of HeLa-PY3-9 and two plates of HFneo10 or from one plate each of HeLa-PY3-9 and HAneo17 were seeded, plated together, and incubated overnight. They were then washed 2 times with PBS and 1ml of 50% polyethyleneglycol (PEG) solution was added. PEG was removed by 5 washes with Phosphate Buffered Saline (PBS). 5 plates of HeLa X HA and HeLa X HF fusions were generated altogether. Fusion hybrids were selected in media containing 200μg/ml of both hygromycin and G418 for four weeks. About fifty colonies were obtained on each PEG treated plate, a total of two to three hundred colonies per revertant type. Thirty individual colonies were then ring-cloned and transfered at low density to 24-well plates and some were subjected to a second ring cloning. The remainder of the original colonies were pulled together and frozen down. The HeLa X revertant hybrid colonies were maintained in hygromycin and G418 containing medium and transfered from 24-well plates to 6-well plates and eventually to 10mm dishes at which point they were switched to medium containing G418 alone. For the anchorage independent growth assay 10^2, 10^3, 10^4 and 10^5 cells of HeLa,HF, HA and six independent clones each of HeLa X HF and HeLa X HA hybrids were plated in soft agar in 35mm dishes. Cells were fed every 3 days. At the end of 3 weeks colonies were stained with 1ml tetrazolium violet overnight, then inverted and dried on 3MM Whatman paper. Colonies were counted from the plates with 10^5 cells plated. The clones were characterized for alkaline phosphatase content and rhodamine retention. The results of these experiments as well as of the soft agar growth assay are reported in Table 1.II.

Antibodies:

Polyclonal antibodies against E7 used for immunoprecipitation and for Western blotting after immunoprecipitation were a generous gift from Denise Gallaway (FHCRC, Seattle) and Elisabeth Schwarz (DKFZ, Heidelberg) respectively. Anti-Rb antibodies C-36 and IF8 were purchased from Santa Cruz Biotechnology; 29C1 was kindly provided by Steve Dowdy (M.I.T.). Anti-HPV-18/E6 antibody C1X1 and anti-HPV16/18 antibody CP15 used respectively for E6 immunoprecipitation and Western blotting were purchased from Oncogene Science and Santa Cruz Biotechnology. For the study of complex formation between p53 and other proteins we used the ImmunoCruz system from Santa Cruz Biotechnology. For Western blotting of p53 we used the monoclonals DO-1 and 1801 from Santa Cruz Biotechnology and Oncogene Science respectively. For p53 immunoprecipitations with metabolically labeled cell lysates the 1801 antibody was used (Oncogene Science; Ab-1). Monoclonal anti-E2FI and anti-cdc2p34 anibodies were both from Santa Cruz Biotechnology. Antibodies against p34cdc2 and anti-SV40 were mouse monoclonals from Santa Cruz Biotechnology and Oncogene Science respectively.

Immunoprecipitations

For immunoprecipitation of E6 we used an E6 lysis buffer (20mM Tris.Cl pH 6.5, 1mM EDTA, 0.25mM EGTA, 0.6% NP-40, 140mM NaCl, 1mM DTT). For the immunoprecipitation of Rb, E7 and E2FI we used the E1A lysis buffer (50mM Hepes pH 7.0, 0.1% NP-40, 250mM NaCl). Aprotinin (30μg/ml), PMSF (50μg/ml), leupeptin (2μg/ml), pepstatin (1μg/ml) and Sodium Orthovanadate (0.1mM; as indicated in figure legends) were added to the appropriate ice cold lysis buffer prior to cell lysis. Cells growing to 70% confluence were washed twice with PBS, then 0.5ml of lysis buffer were added per 100mm plate. Cell lysis was carried out on ice, lysates were removed by scraping and transfered in Eppendorf tubes, then passed through a 22 gauge needle five times. Lysates were centrifuged in a microfuge at 13,000g for 15 minutes and the protein content of the retained supernatant was quantitated using either the Sigma Protein Microquantitation Kit (Sigma Chemicals) or the BCA Protein Microquantitation Kit (Pierce). 0.5mg of protein were used per immunoprecipitation reaction. Reactions were carried out in duplicate in a total volume of 1ml. BSA was added after protein quantitation to a final 2% weight:volume. Lysates were precleared by preincubation with 50μl of protein A/G conjugated agarose beads (Santa Cruz Biotechnology) for 1.5hrs, centrifugation for 30sec at top speed in the microfuge and removal of the supernatant. 1μg of monoclonal antibody was used per reaction or 1μl of the polyclonal 18E7HX1 (anti E7) or 150μl of hybridoma supernatant containing the monoclonal 29C1 (anti-Rb). Antibody incubations were carried out for 2.5-4hrs, then 30ml of the

appropriate agarose bead conjugate were added and incubation continued for another 1.5 hrs. The beads were washed three times with lysis buffer containing protease inhibitors and sodium orthovanadate but no BSA and once with ice cold PBS with protease inhibitors and sodium orthovanadate. 30µl of 2X Laemmli sample buffer (4% SDS, 20% glycerol, 200mM DTT or 2% b-mercaptoethanol, 120mM Tris pH 6.8, 0.002% BPB) were then added and the samples were stored until gel electrophoresis was carried out. For immunoprecipitations of proteins metabolically labeled with ^{35}S-methionine one 100mm plate of each cell line was incubated with 1mCi of Trans Label (ICN; 1000µCi/mmol) in 2ml of methionine free medium for 1.5hr. Samples were normalized for TCA precipitable counts after preclearing of the lysate with Protein A/G Plus Agarose. 7.5X10^6 CPM's were used per reaction in a total volume of 1ml in the appropriate lysis buffer.

Gel Electrophoresis:

Samples were boiled for three minutes prior to loading on a Laemmli gel. SDS-PAGE was carried out in Tris-Glycine buffer system (Harlow, Antibodies a Laboratory Manual) under constant voltage of 80V in either the Mini Protean (BIO-RAD) or a minigel apparatus from CSB Scientific Co. for 2 hr, or at 150V on a Protean Apparatus (BIO-RAD) overnight. Protein standards used were BIO-RAD low molecular weight prestained standards and BIO-RAD wide range standards which were visualized after transfer by treatment of the Western Blot with the Aurodye Kit (Amersham) according to the manufacturer's protocol.

Westen Blotting:

Separated proteins were transfered overnight on a PVDF membrane (Immobilon-P; Millipore). The transfer took place in the cold in the Trans Blotter apparatus (BIO-RAD) under constant voltage at 60Vin transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Upon completion of transfer, membranes were blocked in Blotto (5% milk in TBS-T) for 30min, then incubated with the appropriate antibody dilution in Blotto for 1hr 15min at room temperature, washed twice with TBS-T (pH 7.6; 20mM Tris, 137mM NaCl; 0.1% Tween 20) for 10min at room temperature, incubated with a horseradish- peroxidase-conjugated anti-mouse or anti-rabbit polyclonal secondary antibody (1:2000 dilution in Blotto; Santa Cruz Biotechnology or Transduction Laboratories) for 30min and washed twice with TBS-T and once with TBS (same as TBS-T without the Tween) or PBS. The ECL reagents (Amersham) were used for antigen detection according to the manufacturer's protocol. Membranes were stripped in 0.2M ethanolamine at

82°C for two hours according to the manufacturer's protocol, washed with PBS-0.4% Tween 20 and stained in 0.1% India ink in PBS-0.3%Tween 20 (Harlow book as reference).

Immunoaffinity purification of p53:

p53 protein from HeLa, HA, HF, A431 and Saos-2 cells was purified on a mAb 1801 column (p53 ImmunoCruz, Santa Cruz Biotechnology) according to the supplier's protocol. Cell lysates were normalized for protein content prior to run through the column. The experiment has been repeated 3 times. Protein run through the column ranged from 3-20mg in 2ml of lysate. The final eluate+Laemmli buffer volume was 600ml and 1/12 of that was used per electrophoresis run. When 20mg of lysate were used cell lysates were first concentrated on Centriprep-3 columns (Amicon) prior to passage through the immunoaffinity column.

Retroviral transduction

Retroviral vectors expressing HPV-16 E6, E7 and a hybrid E6/E7 mRNA as well as production of infectious amphotropic virus and infection of cell lines of interest have been described elsewhere (Halbert et al., 1991). Infections were kindly carried out in Denise Galloway's Laboratory. The retroviral vectors LXSN and SS41 which expresses a "supperimmortalizing" mutant of SV40 large T have also been described (Miller et al., Westerman et al., 1996). For the SV40 infections ecotropic helper-free virus was generated by transient transfection of the highly transfectable BOSC cell line (60% transfection efficiency) by a highly efficient precipitation with calcium phosphate method. High titer amphotropic split helper-free retrovirus was generated by subsequent infection of the mouse ΨCRIP packaging cell line. Infectious ΨCRIP supernatant was then incubated with HeLa and revertants for 24 hours. For all infections, infected cells were selected in 500μg/ml G418. The pull of G418 resistant cells was tested for growth in soft agar and molecularly characterized by Western analyses with anti-p53 and anti-SV40 antibodies.

Results

Isolation of revertants:

To increase the probability of isolating non-transformed revertants, HeLa cells were treated with the alkylating agent, ethyl methanesulfonate (EMS). A concentration of 3 mM EMS and an incubation time of 18 h was lethal to approximately 30% of the exposed HeLa cells. After staining with rhodamine 123, the cells were trypsinized and separated by fluorescence-activated cell sorting (Zarbl et al., 1987). The ability of individual colonies to retain the fluorescent dye was examined directly by epifluorescent illumination using an inverted microscope. Twenty-seven colonies that had a persistently low level of dye retention were cloned. Only nine of the colonies survived the initial cloning procedure. These nine clones were recloned twice using cloning cylinders.

The individual clones were then tested for their ability to grow in soft agar. Only two of the clones, designated HA and HF, displayed a reduced cloning efficiency in soft agar (Table 1.1). These two clones were chosen for further characterization. A comparison of the morphologies of these revertant cell lines with the parental HeLa cell line is presented in Figure 1a. In contrast to HeLa cells, the revertant cells had extremely flat and translucent morphologies that were more characteristic of non-transformed cells.

The revertants were next tested for their ability to grow in suspension cultures. Many transformed cells, including HeLa can be adapted to grow in suspension cultures. In contrast, most normal cells must be attached to a solid matrix in order for efficient growth *in vitro*. The growth curves for the revertants and HeLa in suspension culture are illustrated in Figure 1b. As expected, HeLa cells quickly adapted to the new growth conditions, while neither revertant clone grew under these same conditions.

Revertants and HeLa cells were next tested for tumorigenicity after subcutaneous injection into nude mice. Tumors formed in 4 out of the 5 mice injected with HeLa cells. These tumors first became palpable after a latency period of six weeks and grew to a size of about 2 cm by week 12. No tumors were detectable in any of the mice injected with either HA or HF cells even after a period of 22 weeks (Table I).

Tumor suppressor activation is responsible for reversion of both HA and HF.

Stanbridge and co-workers have previously carried out somatic cell hybridization experiments using HeLa cells and and primary human fibroblasts. They have demonstrated that

the short arm of human chromosome 11 harbors a tumor suppressor gene that is able to suppress the tumorigenicity of HeLa cells. In order to determine whether a tumor suppressor had been activated or whether an oncogene had been inactivated in the HeLa revertants we created HeLaXRevertant hybrids. Hygromycin resistant clones of HeLa cells were fused with neomycin-resistant clones of HA and HF revertants. Hybrid clones were selected in media containing both selection agents, were subcloned at least once, then tested for growth in soft agar and retention of rhodamine. Throughout cloning, cells were tested for prolonged rhodamine retention and about 75% of the initial clones showed loss of retention. Six of HeLa X HA and HeLa X HF fusion hybrid clones randomly selected were tested and all of them showed reduced soft agar growth as compared to HeLa. Fusion hybrids are larger than HeLa and revertants and they are more similar in morphology and growth to their revertant parent than to HeLa with the exception of clone HF X HeLa 18.2. Clone HF X HeLa 32 and its subclones have a unique fibroblastic morphology. The results of these experiments are summarized in Table 1.II. Although we have not yet tested our hybrids genotypically by caryotyping, the clones under study were maintained under double selection for long periods of time and subjected to cloning, subcloning and low density plating throughout the duration of the double selection. Furthermore, as confirmed by standard temperature stability and enzymatic assay as well as by a protein gel electrophoresis assay HeLa ATCC CCL2 expresses high levels of the bone/liver/kidney alkaline phosphatase enzyme while our revertants express lower levels of the placental enzyme (data not shown). The hybrid clones of reference in this report were also analyzed at the protein level for their alkaline phosphatase contents and showed either revertant type isozyme profiles or intermediate profiles where both isozymes were present but the BLK levels were greatly reduced. Finally, the clones examined had morphologies distinct from each other's and from the parental HeLa and revertant cells. Thus we do not believe that the clones examined represented co-cultivations of HeLa and revertants. These results indicated that the revertant phenotype is dominant and led us to believe that a tumor suppressor gene has been activated in the revertants.

The E6 and E7 protein levels are unchanged between Hela and revertants

Human papilloma virus is considered a causative agent of cervical cell transformation and continued expression of the E6/E7 genes has been found necessary for the maintenance of the transformed phenotype of the HeLa cell (Bosch et al., 1990; Bosch et al., 1991). Cell lines and carcinomas carrying HPV integrations express hybrid mRNA species that encode for both E6 and E7 proteins, which are then presumably generated by means of differential translational termination (Schwarz et al., 1985; Schneider-Gadicke et al., 1986; Schneider-Gadicke et al.,

1987). Boylan and Zarbl thus examined whether the levels of the E6-E7 mRNA transcript was altered in the revertants and found no quantitative or qualitative changes at the mRNA level (unpublshed data). Immunoprecipitation and immunoblotting experiments showed that the E7 protein levels are also unchanged between HeLa and revertants (Figure). In these experiments, a different antibody was used for immunoprecipitation and for immunoblotting to increase the specificity of substrate recognition. Each experiment was repeated twice. The cervical carcinoma cell lines C4-I and C4-II, which carry integrated sequences of HPV-18 and express the same E6-E7 mRNA transcript as HeLa, were used as positive controls. FS4 or Saos-2 which do not carry HPV sequences and thus do not express HPV E6 and E7 were negative controls cell lines. These experiments have established that the expression of the E6 and E7 dominant oncogenes have not been affected at either the mRNA or protein level, but have not addressed the possibility of E6 or E7 functional inactivation by post-translational modification such as phosphorylation.

The state of p53 in HeLa and revertants

Our cell fusion experiments between HeLa and revertants raised the possibility that a tumor suppressor gene may have been activated in the revertants (see previous result). On the other hand, continued expression of the E6/E7 genes has been found necessary for the maintenance of the transformed phenotype of the HeLa cell (Schwarz et al., 1985; Schneider-Gadicke et al., 1986; Schneider-Gadicke et al., 1987; Bosch et al., 1990; Bosch et al., 1991) and the transforming functions of the "high risk" HPV E6 and E7 are thought to result from their respective binding and inactivation of the p53 and Rb tumor suppressor proteins (Dyson et al., 1989; Munger et al., 1989; Munger et al., 1989; Werness et al., 1990). This functonal re-activation of either Rb or p53 tumor suppressors could have occured during the reversion process. Functional re-activation could have resulted from a change in a regulator of either the tumor suppressor or the relevant HPV oncoprotein, leading to the interruption of the association between viral oncogenes and cellular tumor suppressor genes. We have thus examined the state of the p53 and Rb tumor suppressor genes in HeLa and revertant cells.

Previous reports found the levels of p53 protein in HeLa undetectable or negligible (Matlashewski et al., 1986; Scheffner et al., 1991). However, we were able to develop a protocol that allowed us to detect p53 in HeLa and revertants by Western blotting with the anti-p53 antibody DO-1 with or without prior purification of p53 on an immunoaffinity column (ImmunoCruz system, Santa Cruz Biotechnology, mAb 1801). We attribute the increased sensitivity of detection of p53 protein in our Western blotting technque, the DO-1 antibody, which had a better affinity to blotted p53 than any other antibody that we have tested, and use of the ECL chemilluminescent detection system (Amersham) and an excellent secondary goat anti-

mouse IgG polyclonal antibody (Transduction Laboratories). Immunoaffinity purification and Western blotting with DO-1 also allows detection of HeLa p53 even when chromophoric detection by alkaline phosphatase is carried out.

While the levels of p53 in all three cell lines varied depending on cell culture confluence and nutrient availability, both revertants consistently showed an approximatelly five-fold higher level of p53 protein expression in the revertants rather than Hela (Laser Densitometry of Western Blots; average of four independent experiments). Remarkably, in the studies of Scheffner et al. (1991), where HeLa cell p53 content was characterized as insignificant the quantitative difference between p53 content in HeLa and a positive control cell line was only about five -fold (relative values of 0.4 and 2). The HA and HF expressed levels of p53 that were intermediate between those of positive control primary human fibroblasts (FS4) and HeLa cells. Figure 3 shows two immunoblots of p53 in HeLa, revertants and control cell lines indicating their relative levels. Table 1.III summarizes the results of five independent experiments.

In conlusion, experiments that investigated the state of p53 in HeLa and revertant cells using sensitive chemilluminescence detection methods showed that despite expression of E6 in all three cell lines, p53 was expressed at detectable levels Although the fold- differences in p53 levels between HeLa and revertants varied cell culture confluence or nutrient availability, p53 expression was consistently found to be increased in revertants as compared to HeLa. It is possible that increased p53 levels contribute to the revertant phenotype of both or one of the HA and HF cell lines, possibly as a result of inactivation or inhibition of viral E6 oncoprotein function.

p53 associates with cdc2-p34 in HeLa and revertants:

p53 has been previously reported to bind cdc2-p34 in tumor cell lines. Immunoblotting with an anti-p34cdc2 antibody after purification of p53 complexes on an immunoaffinity column is shown in Figure 6. p34cdc2 was readily detectable in association with p53 in HeLa, HA and A431 cells, was detectable after prolonged exposure in HF cells (not shown) and was not seen in negative control Saos-2 cells which do not express p53. Western blot analysis showed that HF cells expressed greatly reduced levels of the p34cdc2 protein (data not shown). Could such a reduction in p34-cdc2 explain the decrease in complexes in HF revertant cells. Even so, the dramatic decrease in p53-cdc2 complexes would be expected to lead to greatly impaired physiological function of this complex in the HF revertant.

The state of the Rb protein in HeLa and revertants

Like p53, Rb is a common target of DNA virus oncogenes namely the SV40 large T, Adenovirus E1A and "high risk"-HPV E7 and its normal tumor suppressor functions are abbrogated (Schmieg et al., 1984; Ludlow et al., 1989; Munger et al., 1989; Kaelin et al., 1990) by preferential binding of these dominant viral oncogenes to underphosphorylated Rb. As discussed in previous sections of this report, we reasoned that Rb activation may have contributed to the revertant phenotype. It has been shown by others that the HPV-16 and -18 E7 proteins decrease Rb levels in transfected human foreskin fibroblasts and epithelial cells (keratinocytes) (Halbert et al., 1991). Thus we further considered the possiblty that E7 functional inactivation in either revertant could lead to elevated Rb levels in this revertant as compared to HeLa. To investigate the state of Rb in HeLa and revertants we carried out immunoprecipitation with the monoclonal antibody 29C1 followed by immunoblotting with the monoclonal antibody IF8. We detected no significant differences between the steady state levels of Rb protein in the three cell lines (Figure 4a). Immunoprecipitation of metabolically labeled proteins (Figure 4b), however, suggested that differences in the synthesis and the state of Rb phosphorylation may in fact exist among the three cell lines. Specifically, increased levels of metabolically labeled Rb were immunoprecipitated in HF cells as compared to HeLa. Furthermore, in both revertants, Rb protein appeared as a doublet of well separated, distinct, phosphorylated and underphosphorylated species, while in HeLa cells the predominant immunoprecipitated species was the underphosphorylated Rb. Underphosphorylated Rb is preferentially bound by E7 (Dyson et al., 1989; Munger et al., 1989; Imai et al., 1991; Munger et al., 1992), but there are no reports on the effect of the E7-Rb interaction on Rb phosphorylation. If Rb-E7 binding interferes with Rb phosphorylation, it is conceivable that the presence of differentially phosphorylated Rb species could result from and be indicative of disrupted Rb-E7 binding and a consequent gain of Rb function in the HF revertant. Alternatvey, differential Rb phosphorylation could be the result of a mutation in Rb that allows the tumor supressor to escape inactivation by the E7 protein. Alternatively, an E7 or Rb regulator i.e. casein kinase II (Firzlaff et al., 1989; Barbosa et al., 1990; Firzlaff et al., 1991) or a cyclin/cdk complex (Taya et al., 1989; Thomas et al., 1991; Hinds et al., 1992; Dowdy et al., 1993; Yen et al., 1994; Zhang et al., 1994) may have been the target of the mutation(s) that generated the HF cell. Functional inactivation of E7 could explain the apparent increase of metabolically labeled Rb in the HF revertant. The fact that the levels of Rb appear similar in immunoprecipitation-immunoblotting experiments and vary in immunoprecipitation from metabolically labeled cells, could be attributed to differences in cell culture confluence or to differences in nutrient and growth factor availability. It is conceivable that the distribution of each cell population -and hence its respective Rb content- varied between

experiments with respect to cell cycle stage. Rb phosphorylation could have also been a function of the respective cell cycle distribution of each cell line.

During the course of unrelated experiments, we found that the differences in Rb phosphorylation between HeLa and revertants were reversed by transient ectopic expression of the adenoviral homologue of E7, the viral oncogene E1A (Figure 4b). E1A can thus change the state of Rb expression and phosphorylation in the revertants to resemble that seen in the HeLa cell. It would therefore be interesting to study whether Rb phosphorylation state and E1A expression induce retransformation of either of the revertants. While E7 expression is known to decrease Rb levels, E1A ectopic expression during transient transfection increased Rb in all three cell lines. It is thus likely that there are fine significant differences between E1A and E7 function. Thus the results obtained after transfection with E1A results may not be strictly indicative of E7 functional inactivation in either revertant.

The revertants differ from HeLa in terms of E2FI binding to Rb

Association between Rb and viral oncogenes prevents Rb binding to E2F a transcription factor involved in control of cell proliferation (Sarnow et al., 1982; Bagchi et al., 1990; Pietenpol et al., 1990; Chellappan et al., 1991; Nevins et al., 1991; Pietenpol et al., 1991; Hiebert et al., 1992; Nevins, 1992; Nevins, 1992; Hiebert, 1993; Morris et al., 1993). Immunoprecipitation of metabolically labeled Rb from HeLa and revertants suggested that differences in Rb phosphorylation may exist between HeLa and revertants. Since phosphorylation regulates Rb-E2F binding throughout the cell cycle (Chellappan et al., 1991; Chittenden et al., 1991; Helin et al., 1992; Shirodkar et al., 1992) and is in general thought to play a pivotal role in the regulation of Rb tumor suppressor functions (Mittnacht et al., 1991; Mittnacht et al., 1991; Cobrinik et al., 1992; Hatakeyama et al., 1994; Mittnacht et al., 1994), I reasoned that differences in Rb phosphorylation between HeLa and revertants may lead to corresponding differences in Rb-E2F binding. I have thus carried out immunoprecipitation of E2FI containing complexes from Hela and Hela revertant lysates with an anti-E2FI antibody, followed by immunoblotting with an anti-Rb-antibody. This experiment revealed Rb-E2FI binding in the HF revertant and to a lesser extent in the HA revertant. Such binding was not present in the HeLa cell (Figure 1.6a). Subsequent incubation of the supernatant from the E2FI immunoprecipitation with anti-Rb antibody immunoprecipitated unbound Rb from the HeLa lysate. A smaller amount of unbound Rb was immunoprecipitated from the HA lysate (Figure 1.6b). Western blotting of nuclear extracts from Hela, HF and HA showed that E2FI is expressed in Hela but was not detectable in the revertants (data not shown). Taking into account the state of Rb in the three cell lines discussed earlier in this report, the results shown in Figure 1.6 suggest that differential Rb regulation and/or E7

functional inactivation may have stabilized the physical interaction of Rb and E2FI. Rb-E2FI association may in turn down-regulate the proliferative functions of E2F in the revertants.

Ectopic expression of viral oncogenes

The retroviral vector LXSN (a gift of D.A.Miller, FHCRC, Seattle) was used to generate all of the viral oncogene transducing vectors. LXSN contains the Moloney Murine Leukemia (MMLV) Virus Long Terminal Repeat (LTR) Promoter, a polylinker, the ampicillin resistance gene and bacterial replication origin for expression in bacteria, and the neomycin gene under the SV40 enhancer. Retroviral infection results to a single integration of provirus in each cell. Each infected cell contains an integration,each integrated copy drives the expression of a G418 resistance gene as well as of the gene of interest. Hence while stable transfection requires the cloning of single cells and characterization of individual clones, a pool of G418 resistant cells can be used after retroviral infection and integration. Given the high copy number of HPV-18 DNA integrated in the revertant genome, and the dominance of the revertant phenotype in HeLa x revertant hybrids we did not expect that retroviral transduction of either or both HPV oncogenes would affect the growth properties of the revertants in soft agar. Our results have been consistent with this line of reasoning as shown in Table 1.IV. When revertants were infectedwith defective, helper-free retroviruses that expressed high levels of the HPV-16 E6 , E7 or E6 and E7 oncogenes under the control of the strong promoter present in the Large Terminal Repeat (LTR) (Halbert et al., 1991; Foster et al., 1993). Since the retroviral constructs also expressed the bacterial neomycin resistance gene, we examined the phenotype of G418 resistant clones that arose after infection. The morphology of the individual clones was indistinguishable from those of the parental HA and HF revertants. We therefore tested pools of infected HA and HF cells for their ability to grow in semi-solid medium (Table III). The results indicated that infection with retroviruses expressing the E7 oncogene did increase the cloning efficieny of the HF revetant in soft agar medium significantly, suggesting that decreased levels of functional pRB may contribute to the revertant phenotype of this clone. In this experiment the cloning efficiency of the HA clone was sunstantially higher than that observed in previous experiments. However. it should be noted that the former experiments were performed several years earlier, thus suggesting that the HA clone may have acquired a more aggressive in vitro growth phenotype during continuous passage. Despite the observed effects of the E7 oncogene and enhanced cloning efficiency of the HA clone, our results also demonstrated that ectopic overexpression the E6, E7 or E6/E7 oncogenes in the revertants failed to restore the transformed the *in vitro* phenotypes characteristic of the parental HeLa cells. In contrast, expression of a superimmortalizing mutation of SV40 Large T in the HA revertant was able to retransform this cell line.

Discussion

Selection of non-transformed revertants based on loss of the rhodamine 123 has been used successfully to isolate revertants from populations of oncogene transformed fibroblasts (Zarbl et al., 1987; Maniatis et al., 1982). The present study describes the use of the same selection procedure to isolate non-transformed revertants from HeLa cells that were derived from a human cervical carcinoma. Initial phenotypic characterization of the revertants demonstrated that these cells lost most of the *in vitro* properties associated with transformed cells, including a transformed morphology, clonigenicity in semi-solid medium, and growth in suspension culture. In these respects the revertants differ from HeLa x normal human fibroblasts hybrids, which, although non-tumorigenic in nude mice, maintain the *in vitro* properties characteristic of the parental HeLa cell (Maniatis et al., 1982; Srivatsan et al., 1986; Stanbridge et al., 1986). However, the revertants isolated in our study also failed to form tumors in nude mice.

In order to rule out the possibility that the revertants represented contamination with other cell lines, limited DNA fingerprinting of HeLa cell and revertant cell DNA was performed using four variable number terminal repeat (VNTR) probes (data not shown). The fact that the revertants were in fact derived from HeLa cells was further confirmed by Southern hybridization analysis (data not shown) of the integrated HPV-18 sequences, which unequivocally demonstrated that the three cell line had identical viral genome integration sites. The results of this analysis further indicated that the integrated viral genomes remain intact in the revertants. Northern hybridization analyses (data not shown) indicated that the HPV-18 specific transcripts are expressed at the same level in the revertants as in the parental HeLa cells. The two major transcripts identified by Northern hybridization correspond in size to mRNA species identified by other researchers (Inagaki et al., 1988; Schwarz et al., 1985). The smaller 1.6 kb transcript was shown to contain an alternatively spliced E6 gene and a full length E7 gene. The larger 3.4 kb transcript was also polycistronic and encoded full length E6 and E7 genes and most but not all of the E1 gene (Inagaki et al., 1988). The oncogenic potential of the HPVs has been attributed to the E6 and E7 open reading frames (Dyson et al., 1989; Crook et al., 1991; Munger et al., 1989; Werness et al., 1990). I have demonstrated that the levels of E6 and E7 proteins expressed in the revertants are unchanged relative to the parental HeLa clone. Since HeLa cells contain multiple copies of HPV-18 DNA integrated into their genome, it is not likely that mutational inactivation of the E6 or E7 genes has lead to the revertant phenotype (Lazo et al., 1987; Schwarz et al., 1985) This interpretation is consistent with our cell fusion experiments which indicate that the revertant phenotype is dominant in HeLa x revertant hybrids and ectopic excression of the viral oncogenes failed to retransform. Although we cannot formally rule out the presence of dominant negative mutants of the E6 or E7 proteins, the involvement of such mutants in the reversion process is

unlikely, given the high copy number of the HPV-18 genome. It is thus more likely that the revertant phenotype resulted from mutations that activate a suppressor of cell transformation. Stanbridge and co-workers have demonstrated that suppression of tumorigenicity in HeLa X fibroblast hybrid cells correlated with the presence of chromosome 11 and could be conferred to HeLa by microinjection of the short arm of human chromosome 11. Zur Hausen an co-workers have used Stanbridge's hybrids as well as hybrids between HeLa and immortalized rodent cells to show that continued E6 and E7 function, are required for the maintenance of the transformed phenotype (Bosch et al., 1990; Bosch et al., 1991). These hybrids continued to express E6 and E7 when grown in culture and grew in soft agar but mRNA levels of the viral oncogenes declined two days after transplantation into nude mice and consequently failed to induce tumors. Zur Hausen and coworkers have further shown that the suppression of Hela cells tumorigenicity in athymic mice correlates with a dramatic decline of HPV E6 and E7 expression post-injection. The fact that our revertants have lost the *in vitro* properties of transformed cells indicate that the suppressor activated is probably not the chromosome 11 suppressor and that it may be able to bypass the E6 and E7 functional pathways. Furthermore, all of the non-tumorigenic cell hybrids generated from the fusion of HeLa cells and normal human fibroblasts invariably maintained the *in vitro* characteristics of the transformed parental Hela . Thus, loss of the tumor suppressor gene on chromosome 11 is likely to be associated with tumor progression rather than initiation of carcinogenesis. In contrast, the events that led to reversion in our system may have affected early pathways of transformation. It has been suggested that oncogenic HPVs are risk factors that predispose cells to secondary events, including activation of a dominant oncogene and/or loss of additional suppressor genes, necessary for progression of cells to a malignant phenotype (Vousden et al., 1989; zur Hausen et al., 1989). In other words, the E6 and E7 functional pathways contribute to the early events of carcinogenesis and in our system they may have been affected during the reversion events despite the lack of evidence for mutation within the revertant E6 an E7 genes. Changes in the association between one of the viral oncogenes and its respective tumor suppressor or gain of function of either Rb or p53 would be the equivalent of partial or complete interruption of E6 and E7 function. We thus studied the associations of E6 and E7 with Rb and p53 as well as changes in Rb and p53 properties that would indicate gain of function in the revertants.

Genetic alteration of p53 is the most common known event in human oncogenesis (Levine, 1994). A vast volume of experimental data indicates that p53 functions as a negative regulator of cell proliferation and its inactivation is necessary for the development of malignancy (reviewed in (Zambetti et al., 1993), still the mechanism through which p53 functions as a tumor suppressor remains obscure. Wild-type p53 has distinct DNA binding and transcription factor properties (Kern et al., 1991; Kern et al., 1992; Zambetti et al., 1992). p53 expression is inducible

by DNA damaging agents such as Actinomycin D and γ-irradiation and followed by the transcriptional activation of genes involved in DNA repair such as gadd45 (Kastan et al., 1992 ; Tishler et al., 1993). Overexpression of p53 results in G1 arrest (Kastan et al., 1992; Slichenmyer et al., 1993) or apoptosis (Lowe et al., 1994; Lowe et al., 1993a; Lowe et al., 1993b; Lowe et al., 1993c)and p53-transfection in p53 minus fibroblasts results in G1 arrest upon treatment with DNA damaging agents (Nelson et al., 1994; Kastan et al., 1991; Sun et al., 1986; Lowe et al., 1993). Taken together, these results support a model that p53 functions to prevent DNA replication upon DNA damage until repair can occur, or induces apoptosis if repair or replication fails (reviewed in Lane, 1993; Lane, 1992). Although selection against wild-type p53 concerns the genotype of a very small fraction of cervical carcinoma, inactivation of the the tumor suppressor protein by the "high risk" HPV E6 in all remaining tested cases has so far implicated wild-type p53 function with one hundred percent of all the cervical carcinoma tumors and cell lines examined (Scheffner et al., 1991; Crook et al., 1992a; Crook et al., 1992b; Crook et al., 1991). The major mechanism of control of p53 expression appears to be through protein stability (post-transcriptional modification; Oren et al 1981, Reich et al. 1981). Since HPV-16 and -18 could bind E6 *in vitro* and cervical carcinoma cell lines with HPV integrations carry the wild-type p53 gene and express the p53 mRNA but show low levels of p53 protein, the hypothesis that HPV E6 targets p53 for ubiquitination has been tested. HPV -16 and -18 E6 were thus found to induce ATP and ubiquitin -dependent degradation of p53 in rabbit reticulocyte lysates (Scheffner et al. ,1990) and a novel ubiquitin-dependent pathway has been implicated in p53 degradation (Scheffner et al., 1992; Scheffner et al., 1992; Scheffner et al., 1993). In Hela cells, p53 mRNA expression has been previously reported not to be inhibited but to be comparacle to that of normal cells while the levels of the p53 protein were found to be undetectable (Matlashewski et al., 1986) or very low (Scheffner et al., 1991). p53 cDNA sequence in our parental clone ATCC CCL2 has been reported to be wild-type (Scheffner et al., 1991). p53 protein synthesis was, however, shown to take place when p53 protein present was stabilized by transfection with a mutant p53 and detected by immunoprecipitation (May et al., 1991). The result was consistent with the hypothesis that p53 protein in the HeLa cell is targeted for rapid degradation by the HPV-18 E6. Sensitive chemilluminescence detection used in immunoblots in combination with high affinity primary and secondary antibodies gave us the ability to easily detect p53 in HeLa by Western, or a combination of immunoaffinity purification and immunoblotting. When HA and HF steady state p53 levels were compared to HeLa we found that p53 levels were increased 5-fold in the revertants. Elevation of p53 levels in the revertants could have resulted from a stabilizing mutation in the p53 gene. We believe, however, that this cannot be the case for both revertants. Increased stability p53 mutants are usually gain of function mutants that actually induce cell proliferation. In fact p53 was first identified in mutant form and characterized as an

oncogene. It is thus counterintuitive -though always possible- to assume that the p53 in the revertants carries a stabilizing mutation. We are currently ruling out the possibility of p53 mutation using direct sequencing of p53 cDNA from HeLa and revertants and have data to suggest that revertant express the wild-type p53 gene. Further study of our system could also be helpful in identifying factors that are involved in p53-mediated G1 arrest or apoptosis.

Complex formation between p34cdc2 and p53 was seen in in Sv40 transformed mouse cell lines (Milner et al., 1990 0. The same report showed that such interactions are specific for wild-type p53. Other investigators have shown that p34cdc2 is a modulator of p53 phosphorylation both *in vitro* and *in vivo* and phosphorylation of p53 at the p34cdc2 site has been found necessary for the ubiquitin-dependent degradation of the p53 protein (Lin and Desiderio, 1993). We are currently investigating whether increased p53 levels in the HF cell line, may result from the lack of p53-p34cdc2 complex formation, and from a subsequent failure to target 53 for degradation by phosphorylation at Ser-315. It remains unclear whether by binding p34cdc2 p53 can also regulate its function. The involvement of cdc2 in the reversion of the HF cell line is also being investigated.

Immunoprecipitation of Rb from metabolically labeled cell extracts suggested different rates of Rb synthesis and phosphorylation in the three cell lines. Specifically, Rb levels appeared lower in HA than in HeLa or HF, Underphosphorylated Rb was the predominant Rb form in HeLa while two distinct bands were present in HA and HF. We suspect differences in the Rb phosphorylation potential of revertants and are currently investigating this hypothesis. Rb changes in phosphorylation may account for the co-immunoprecipitation of Rb with E2FI from revertant cell lystates that suggests Rb activation. The extent/dose of functional Rb activation may be important to Rb contribuion in the reversion process as may be the interplay between Rb and p53 gain of function in the revertants. E2F1-Rb association is transient in the cell cycle and dependent on Rb phosphorylation by cell cycle regulatory complexes (Schwarz et al., 1993; Shirodkar et al., 1992; Hiebert et al., 1993; Nevins et al., 1992). We are currently investigating whether complex formation between Rb and E2F-1 could account for differential E2F-1 activity in the revertants and what changes in Rb or E2F regulation can account for the restoration of complex formation in HF. We are also testing the hypothesis that gain of function of Rb through this complex may have contributed to the reversion (Hiebert et al., 1993).

Retroviral integration and ectopic expression of HPV-16 E6, E7 or a combination of the two had no effect on the revertant phenotype suggesting that the reversion event has bypassed the E6 and E7 functional pathways. Ectopic expression of SV40 Large T antigen, however, increased the clonigenicity in soft agar of the HA but not the HF cell line by one hundred-fold. Hence SV40 large T possesses a biochemical function that can override the mechanisms responsible for reversion in the HA, but not the HF cell line.

In conclusion, we have shown that protein-protein interactions in the pathway of p53 and/or Rb are affected in these two independent revertants of the HeLa cell line. The finding provided evidence that change along the p53 with or without activation of the Rb pathway may respectively be an obligatory and a necessary occurence for the reversion of cervical transformation. These results corroborate functional studies which by retroviral transduction of viral oncogenes suggest that the E6 and/ E7 pathways have been interrupted/bypassed during the reversion process.

Table 1.I: Clonigenicity and Tumorigenicity of Revertants

	Clonigenicity		Tumorigenicity	
Cell Line	Anchorage Dependent Clonigenicity *in vitro* (%)	Clonegenicity in Semi-Solid Medium (%)[a]	Number cells injected	Number of mice with tumors
HeLa	50%	20%	1×10^7	4/5
HA	5%	0.01%	1×10^7	0/4
HF	25%	0.2%	1×10^7	0/4

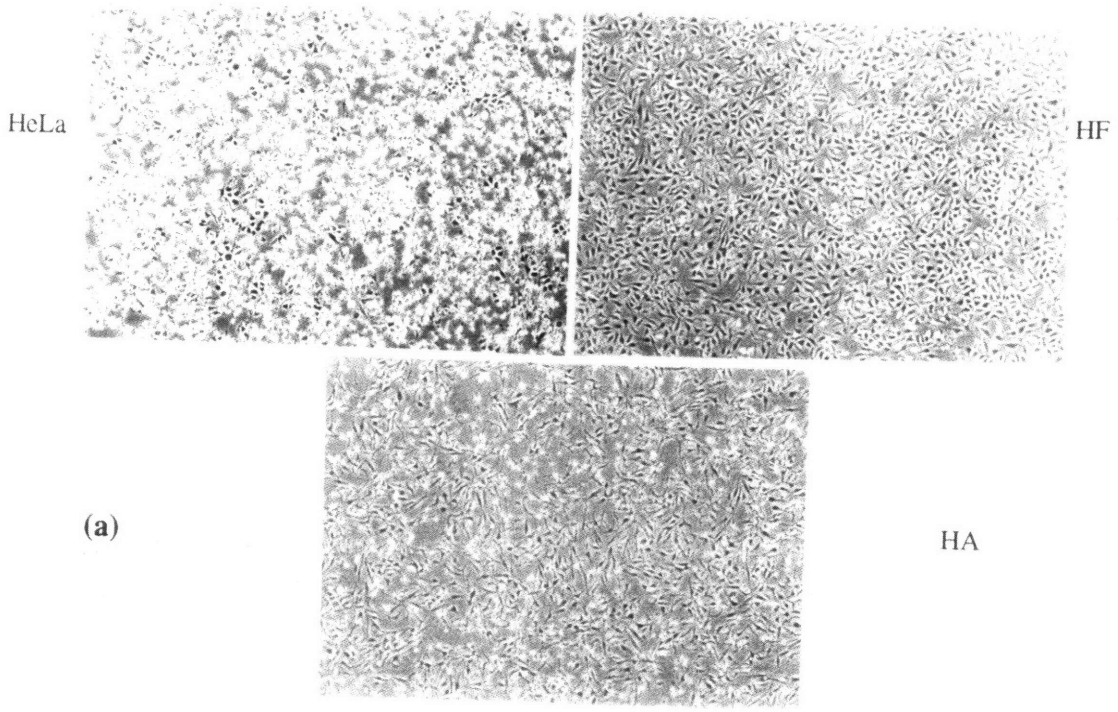
[a]Growth in soft agar was performed in triplicate for each cell line tested. Exponentially growing cells were removed from dishes by trypsinization and diluted with MEMN + 10% FBS. Aliquots containing 10^4 cells, 10^3 cells or 10^2 cells were removed and seeded into molten Agar. The number given is the average number of colonies formed per 100 viable cell seeded. Viability was determined by seeding an identical dilution of cells onto standard tissue culture dishes.

Table 1.II: Clonigenicity of Hela cells, HA and HF revetants and hybrid cells in soft agar.

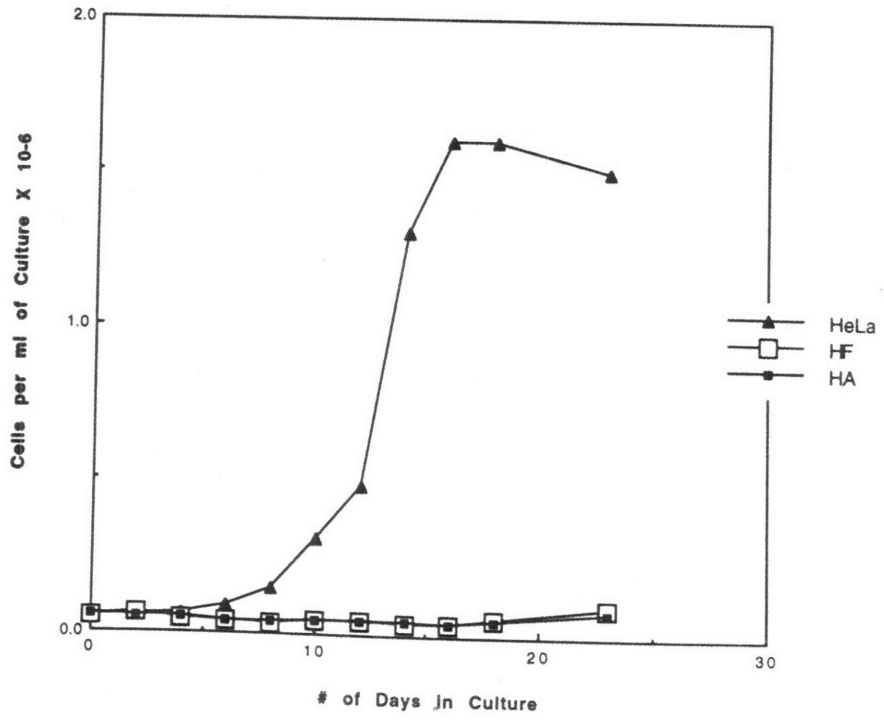
CELL LINE	CLONING EFFICIENCY IN SOFT AGAR (%)[a]	RHODAMINE RETENTION
Hela	20	PROLONGED
HA	0.003	NORMAL
HF	0.05	NORMAL
[illegible]	[illegible]	ND
[illegible]	[illegible]	PROLONGED
[illegible]	[illegible]	PROLONGED
[illegible]	[illegible]	NORMAL
[illegible]	[illegible]	NORMAL
[illegible]	[illegible]	ND
HF X HeLa 32	0.005	ND
HF X HeLa 33	<0.001	NORMAL
HF X HeLa 8	0.003	ND
HF X HeLa 18.2	0.2	PROLONGED
HF X HELA 20	<0.001	ND

Growth in soft agar was performed in triplicate for each cell line tested. Exponentially growing cells were removed from dishes by trypsinization and diluted with MEMN + 10% FBS. Aliquots containing 10^5 cells, 10^4 cells or 10^3 cells were removed and seeded into molten Agar in 30mm dishes. Reduction of cells seeded by a factor of 10 resulted in reduction of colonies by about between 50- and 100-fold.

Figure 1


HeLa
HF
HA

(a)


2.0
1.0
0.0
0
10
20
30
Cells per ml of Culture X 10-6
# of Days in Culture
HeLa
HF
HA

(b)

Figure 1.1: Morphology of Revertant Cell Lines and Growth Curves of Cell Lines Grown in Suspension

(a) Top : Rounded and refractile morphology of the transformed parental HeLa cell line. Middle: Flat, translucent, nontransformed appearance of revertant clone, HA. Bottom: Flat, translucent, nontranformed appearance of revertant clone, HF. Magnification 40X; phase contrast optics.
(b) Cells in suspension cultures were grown in spinner flasks and incubated at 37^{o} c in a 5% CO_2 atmosphere on top of magnetic stir plates as described in Materials and Methods. Two identical cultures for each cell line were grown in parallel. Plotted on the graph above is the average number of cells per ml measured for each culture versus the number of days in culture.

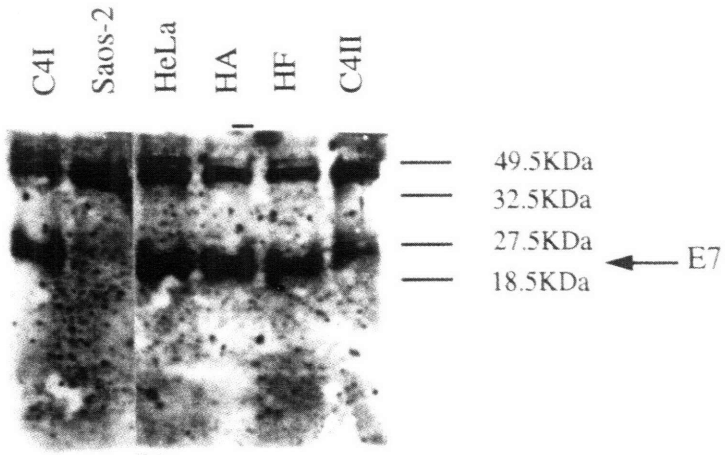
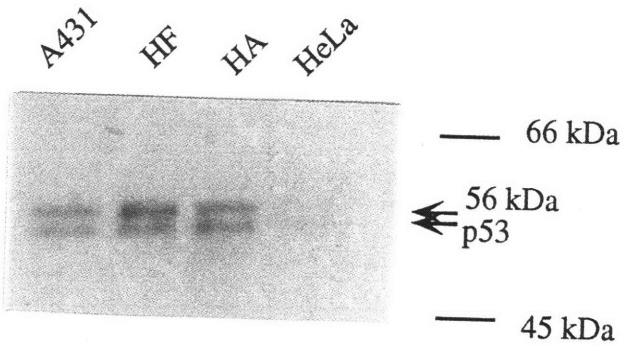

C4I
Saos-2
HeLa
HA
HF
C4II
49.5KDa
32.5KDa
27.5KDa
18.5KDa
E7

Figure 1.2 : The E6 and E7 protein levels are unchanged between Hela and revertants

(a) Immunoblot showing the 18KDa E6 species and a higher molecular weight species recognized by the anti-HPV16/18-E6 monoclonal antibody CP15. E6 and associated proteins were immunoprecipitated by anti-HPV18-E6 monoclonal antibody C1X1. 15% SDS-PA mini gel run at 80V for 2hr. The HeLa lane is overloaded. When normalized for loading, the E6 in all three cell lines is the same. The secondary antibody was horseradish-peroxidase-conjugated (Signal Transduction Laboratories) and the ECL reagents (Amersham) were used for visualization of bands.

(b) Immunoblot showing the 21KDa E7 species recognized by an anti-HPV18-E7 polyclonal antibody (gift of Elizabeth Schwarz). E7 and associated proteins were immunoprecipitated by anti-HPV18-E7 polyclonal antibody (gift of Denise Gallaway). 15% SDS-PA mini gel run at 80V for 2hr.


A431
HF
HA
HeLa
66 kDa
56 kDa
p53
45 kDa

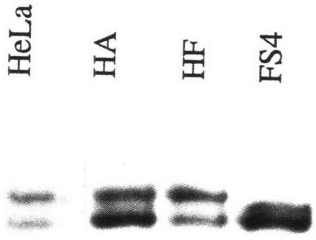

HeLa
HA
HF
FS4

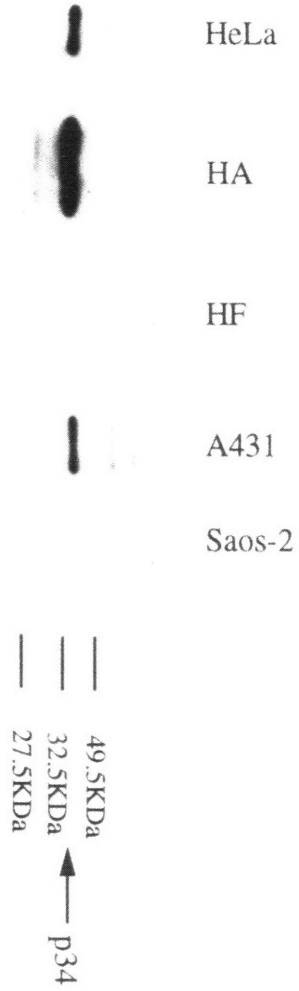
Figure 1.3: The state of p53 in HeLa and revertants.

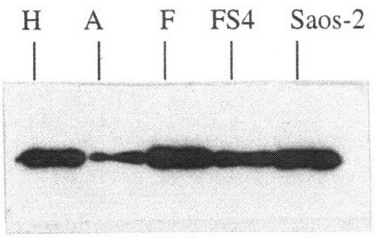
(a) Western blot of p53 following immunoaffinity purification as described in Materials and Methods. The secondary antibody was alkaline-phosphatase-conjugated (Santa Cruz Biotechnology). No phosphatase inhibitors were used in this experiment. A 20cm X 20 cm 10% Laemmli SDS-PAGE gel was run for 12hr at 4°C and 0.35Amp. BCNP/NBP tablets (Sigma) were used as substrate for this experiment and the blot was washed with Tris.Cl, NaCl prior to incubation with the enzyme substrate. In order for the signal from HeLa to be detectable we loaded 3X more protein from HeLa than from revertant cell lines in this experiment.
(b) Western blot of p53 without immunoaffinity purification. The secondary antibody was horseradish-peroxidase-conjugated (Signal Transduction Laboratories) and the ECL reagents (Amersham) were used for visualization of p53. Phosphatase inhibitors were included in the protein extraction buffer. 50μg of protein were loaded per lane on a 20 X 20 cm, 10% Laemmli SDS-PA gel which was run overnight at 6°Cat 80V.

Table 1.III: Relative levels of p53 protein in HeLa and revertants

Relative p53 protein levels (w/ respect to HeLa)	**Cell line**
1	HeLa
5 + 3	HA
5 + 3	HF
13 + 10	FS4
0	Saos-2

Table III: Relative levels of p53 protein from Western immunoblots were quantitated by scanning laser densitometry of linear film exposures using an LKB Laser densitometer. To obtain the relative p53 levels readings for the intenity of the p53 bands from one autoradiogram were divided by the corresponding value from the HeLa lane. The values presented here are averages of five independent experiments for the HeLa, HA and HF and two independent experiments for the FS4 and Saos-2 cells. No bands were detected in p53 negative control cells Saos-2and the actula densitometer reading for the correponding lane was 0.


HeLa
HA
HF
A431
Saos-2
49.5KDa
32.5KDa
27.5KDa
p34



$p34^{cdc2}$

Figure 1.4: p53 associates with cdc2-p34 in HeLa and revertants:

(a) Western blot of p34cdc2 following a immunoaffinity purification of p53 and associated proteins. A 15% mini Laemmli SDS-PA gel was run for 2hrs at 80V.
(b) Western blot of p34cdc2 from nuclear extracts of HeLa, revertants and control cells. A 12% Laemmli SDS-PA gel was run for 2hr at 80V.

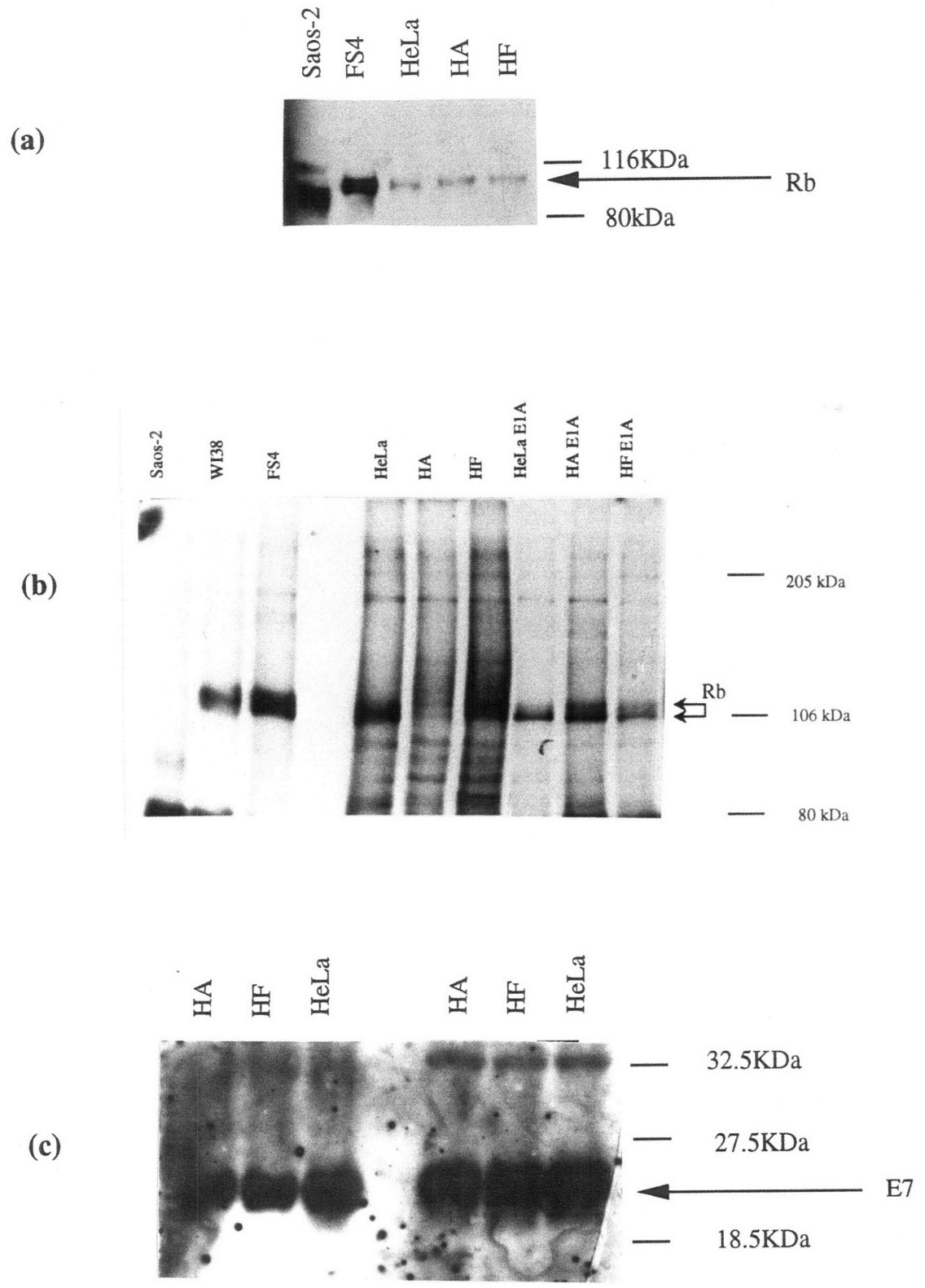

(a)
Saos-2
FS4
HeLa
HA
HF
116KDa
Rb
80kDa
(b)
Saos-2
WI38
FS4
HeLa
HA
HF
HeLa E1A
HA E1A
HF E1A
205 kDa
Rb
106 kDa
80 kDa
(c)
HA
HF
HeLa
HA
HF
HeLa
32.5KDa
27.5KDa
E7
18.5KDa

Figure 1.5: The state of the Rb protein in HeLa and revertants

(a) Immunoblot showing Rb recognized by the anti-Rb IF8 monoclonal antibody. Rb and associated proteins were immunoprecipitated by anti-Rb monoclonal antibody 29C1 (gift of Steve Dowdy, M.I.T). 6% SDS-PA mini gel run at 80V for 2hr.

(b) Immunoprecipitation of Rb-1 from metabolically labeled cell extracts. HeLa, HA and HF cells were either untransfected or transiently transfected with p1Aneo (E1A under the MMLV-LTR) and harvested 24hrs after transfection. Saos-2 cells were used as a negative control and FS4 and WI38 cells are two positive control cell lines. MAb29C1 immunoprecipitates both phosphorylated and unphosphorylated forms of Rb. E1A protein levels were also higher in HA than in HeLa and HF (as determined by immunoprecipitation, data not shown).

(c) Immunoblot showing the 21KDa E7species recognized by an anti-HPV18-E7 polyclonal antibody in immunoprecipitates by either an anti-E7 or an anti-Rb antibody. E7 and associated proteins were immunoprecipitated by anti-HPV18-E7 polyclonal antibody. Rb and associated proteins were immunoprecipitated by anti-Rb monoclonal antibody C-36 (Oncogene Science; anti-Rb-1 Ab-1). 10% SDS-PA 20 X20 gel run at 4^{o}C and 80V onernight.

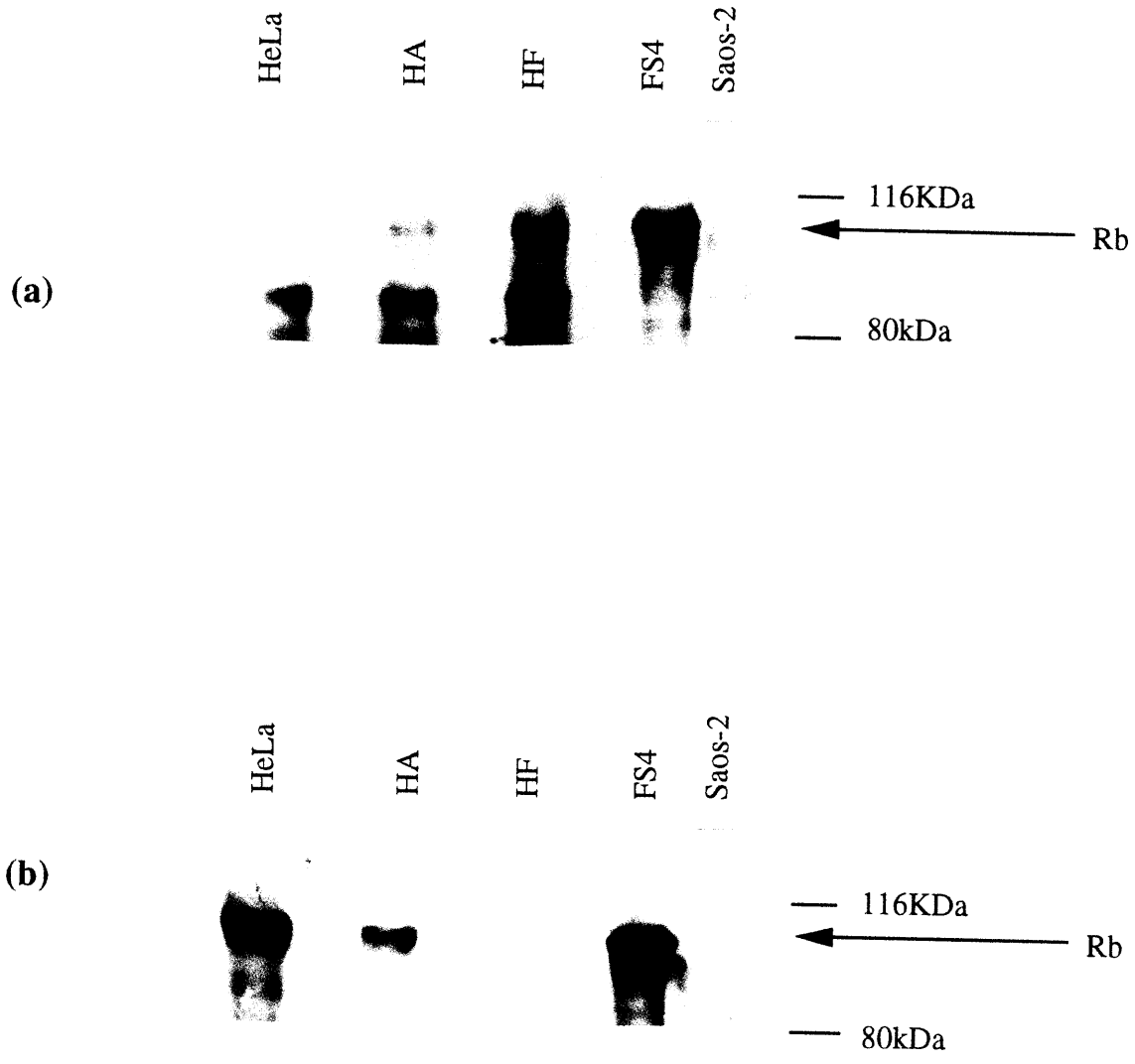

(a)
HeLa
HA
HF
FS4
Saos-2
116KDa
Rb
80kDa
(b)
HeLa
HA
HF
FS4
Saos-2
116KDa
Rb
80kDa

Figure 1.6: E2FI binding to Rb.

(a) Immunoblot showing Rb recognized by the by the anti-Rb IF8 monoclonal antibody in immunoprecipitates with anti-E2F-I monoclonal antibody (Santa Cruz Biotechnology).
(b) Immunoblot showing Rb recognized by the anti-Rb IF8 monoclonal antibody. Rb and associated proteins were immunoprecipitated by anti-Rb monoclonal antibody 29C1, from lysates that had been precleared with the anti-E2F-1 antibody as shown in (a).
Both (a) and (b)6% SDS-PA mini gel run at 80V for 2hr.

Table 1.IV. Soft Agar Cloning Efficiency of Revertants Expressing Ectopic HPV-16 Oncogenes.

Cell Line	Cloning efficiency (%) [a]
HeLa	60
HA + LXSN	< 3
HA + E6	< 2
HA + E7	< 2
HA + E6/E7	< 2
HA + SV40	20
HF + LXSN	< 0.1
HF + E6	< 0.4
HF + E7	< 1
HF + E6/E7	< 1
HF + SV40	<1

[a] Exponentially growing HA and HF cells infected with LXSN retroviruses carrying the denoted HPV-16 oncogenes or vector LXSN alone were harvested by trypsinization and diluted with MEMN + 10% FBS. Aliquots containing 10^4 cells and 10^3 cells seeded into 0.3% molten agar medium in 30mm dishes. Numbers shown are the averages of two independent experiments, each performed in duplicate.

CHAPTER TWO

Inactivation of E6 function and ifferential targeting of p53 for proteolysis leads to p53 activation in two independent revertants of HeLa transformation.

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Abstract

We have isolated two non-transformed, non-tumorigenic revertants of the HeLa (ATCC CCL2) cell line . Unlike the parental HeLa, the revertants have more than 100-fold lower cloning efficiencies in semi-solid medium, and fail to induce subcutaneous tumors when injected into nude mice. Continuous function of the E6 and E7 viral oncogenes of human papillomavirus has been found necessary for the maintenance of the transformed phemotype in HeLa. We have therefore tested for changes in the expression and function of these viral oncogenes. The main function attributed to HPV E6 is inactivation of the p53 tumor suppressor protein mediated by E6-p53 binding and the subsequent targeting of p53 for degradation. Thus HeLa cells express very low levels of p53. Despite unchanged expression of the HPV-18 E6 mRNAand protein. both revertants show increased p53 protein levels. Furthermore somatic cell fusion experiments suggested that a dominant suppressor of transformation had been activated in both revertants. We have been investigating whether functional activation of the p53 pathway has taken place in the revertants and could contribute to the revertant phenotype.

We used retroviral transduction to ectopically express wild-type p53 in HeLa and to examine whether p53 is sufficient for the suppression of the Hela phenotype. Ectopic expression of the wild-type p53 was shown to decrease the clonigenicity of the Hela cell in soft agar by a hundred fold. Mutation in the p53 gene of the revertants could explain the increased p53 expression in these cells but a mutant p53 would not be expected to contribute to the revertant phenotype. RNAase protection assay and direct sequencing failed to detect mutation in the p53 cDNA's from HeLa and revertants. If the revertant p53 was shown to be functionally wild-type, increased p53 protein levels in the revertants as compared to HeLa would be anticipated to contribute to the revertant phenotype. We therefore tested revertant cells for the manifestation of endogenous wild-type p53 function. As has been shown for the wild-type p53 gene in non-transformed cervical cells, endogenous p53 in the revertants but not in Hela: i) is inducible by treatment with Actinomycin D, an inhibitor of DNA synthesis, and by exposure to γ-irradiation, a DNA damaging agent, ii) possesses constitutive and inducible DNA binding properties and promoter transactivating properties specific to the wild-type p53 gene. Furthermore, differential expression of the endogenous gadd-45 and waf-1genes -which are regulated transcriptionally by p53- is observed between HeLa and revertants. An *in vitro* ubiquitination-degradation system was used in order to investigate the causes of increased p53 protein in the revertants. Remarkably in both revertant cell lines ubiquitin-dependent proteolysis of p53 was found to be impeded in comparison to HeLa. Thus, functional activation of p53 in transcription as well as inhibition of ubiquitin-dependent proteolysis correlate tightly with the reversion of transformation of cervical carcinoma cells. E6-p53 complexes are detectable in Hela but not in the HA and HF revertant.

Inhibition of p53 degradation in the HF revertant may also be caused by interruption of complex formation of p53 with p34cdc2. The lack and presence of this interaction in HF and HA respectively may also explain the inability of SV40 Large T to retransform the former and its ability to do so in the latter.

Introduction

Cervical carcinoma is the second most common gynaecological tumor and claims about 500,000 lives worldwide each year. As many as 90% of cervical carcinomas have been found to contain integrated viral genomic sequences of Human Papilloma Viruses -16, -18, -31, -33 or -39 which have consequently been termed "high risk" HPV's (Durst et al., 1983), (zur Hausen, 1987) ,(zur Hausen, 1991), (Schiffman et al., 1991. In benign and premalignant lesions the HPV DNA exists episomally, while in malignant lesions and cervical carcinoma cell lines HPV DNA is integrated, often in multiple copies, with preservation of the E6 and E7 open reading frames and disruption or deletion of the E1 and E2 genes (Schneider- Gadicke et al., 1988 ; Durst et al., 1987; Popescu et al., 1989; Lazo et al., 1989 ; Sousa et al., 1990). In some cases, integration occurs in the vicinity of oncogenes - usually *c-myc* - and leads to their increased expression (Schneider-Gadicke, 1988). The E6 and E7 genes of the "high risk" HPVs have been implicated as essential factors in cervical carcinogenesis because of their presence and expression in cervical carcinomas and carcinoma cell lines (Baker et al., 1987, El Awady et al., 1987, Schneider-Gadicke and Schwartz, 1986, Schwarz et al, 1985, Seedorf et all 1987, Smotkin and Wettstein, 1986, Roggenbuck et al. 1991). Numerous studies have demonstrated the transforming properties of these two viral proteins (reviewed in Mansur and Androphy, 1993). Most importantly , HPV-16 and -18 E6 and E7 can immortalize primary human keratinocytes (Kaur et al, 1988; Hawley-Nelson etal.. 1989; Munger et al., 1989; Hudson et al., 1990; Barbosa et al., 1991; Halbert et al., 1991; Schiller et al., 1989) and their maintained expression has been reported as both necessary and sufficient for tumorigenicity and full blown transformation (Munger et al. 1989, Bosch et al., 1990, Miyasaka et al., 1991). The transforming functions of the E6 and E7 are thought to result from their respective binding and inactivation of the p53 and Rb tumor suppressor proteins (Werness et al., 1990; Munger et al., 1989; Dyson et al., 1989).

The p53 tumor suppressor gene is the most commonly mutated gene in human cancers, with seventy percent of all tumors and tumor cell lines examined carrying an altered p53 gene sequence. Elevated wild-type p53 expression supresses the transformed phenotype of colon carcinoma, osteosarcoma and breast carcinoma cell lines (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Chen et al. 1991; Cheng et al., 1992; Wang et al., 1993). The exact mechanism or mechanisms through which wild-type p53 functions in tumor/growth supression is not known, although numerous studies have defined a role for p53 in response to DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992; Kessis et al., 1993; Lowe et al., 1993; Lowe et al., 1994; Slebos et al., 1994), cell cycle control (Kastan et al., 1991; Perry et al., 1993; Slichenmyer et al., 1993; Chen et al., 1994; Slebos et al., 1994), transcriptional activation (Kern et al., 1991;

Kern et al., 1991; Zambetti et al., 1991; Kern et al., 1992; Zambetti et al., 1992; Zambetti et al., 1993) and DNA replication (Wang et al., 1989; Friedman et al., 1990; Bargonetti et al., 1991; Bargonetti et al., 1992; Friedman et al., 1993; Reynisdottir et al., 1993; Wang et al., 1993; Moses et al., 1994). A widely accepted hypothesis on p53 function is that whenever DNA damage occurs, p53 protein levels become induced by a post-translational mechanism. The p53 induction forces the offended cell to exit the cell cycle by inducing G1 arrest or apoptosis. Thus when p53 function is unperturbed, cells that cannot replicate properly -because their DNA is damaged or because of insufficient metabolites- either stop dividing or die and cannot develop into cancer cells. Induction of p53 upon DNA damage is followed by p53-specific transcriptional activation of genes involved in repair such as the gadd45 gene (Kastan et al., 1992) as well as genes controlling the cell cycle, specifically the cyclin inhibitor cip-1/waf-1 (el Deiry et al., 1993). Transcriptional activation and DNA binding are specific to wild-type p53 and not mutant p53 (Kern et al., 1991; Kern et al., 1991; Kern et al., 1992;) and the induction of cip-1/waf-1 after γ-irradiation requires the presence of a functional p53 gene (Scott Lowe, personal communication; el Deiry et al., 1993). Thus it is thought that the transcription factor properties of wild-type p53 are essential for its role in cell growth control.

The major mechanism of control of p53 expression appears to be through protein stability (post-transcriptional modification; Oren et al 1981, Reich et al. 1981). Since HPV-16 and -18 E6 could bind p53 *in vitro* and cervical carcinoma cell lines with HPV integrations carry the wild-type p53 gene and express the p53 mRNA but show low levels of p53 protein the hypothesis that HPV E6 targets p53 for ubiquitination has been tested. HPV -16 and -18 E6 were thus found to induce ATP and ubiquitin -dependent degradation of p53 in rabbit reticulocyte lysates (Scheffner et al. ,1990) and a novel ubiquitin-dependent pathway has been implicated in p53 degradation (Scheffner et al., 1992; Scheffneret al., 1992; Scheffner et al., 1993). Ubiquitin-dependent degradation of p53 *in vitro* has been more recently shown to take place in the absence of E6 (Ciechanover et al., 1994). Wild-type p53 is a relatively short-lived protein with a half-life of twenty minutes in non-transformed cells. Endogenous levels of p53 protein are low in certain cell types and vary two - to four-fold between various stages of the cell cycle in p53 expressing cells. It is therefore possible that ubiquitin dependent proteolysis is involved in the regulation of p53 protein expression in non-transformed cells and that E6 analogs may exist in such cells.

p53 has been implicated in one hundred percent of all the cervical carcinomas examined. Ninety percent of carcinomas tested contain HPV integrations and express the E6 oncogene which inactivates p53 by targeting it for degradation. All non-HPV cervical carcinoma cell lines tested express a mutant p53 (Scheffner et al., 1991; Crook et al., 1992; Crook et al., 1992; Crook et al., 1991). Deregulation of p53 function thus appears to be an absolute requirement for cervical transformation.

In order to study cervical cell transformation we have employed an *in vitro* cell culture system comprised of the cervical carcinoma cell line HeLa and two non-tumorigenic variants (revertants), HA and HF. Such systems have been used succesfully to identify tumor the *ras* suppressor gene *k-rev*and transformation effector genes (Boylan et al., 1991; Kho et al., 1991), whose products comprise and/or regulate the biochemical pathways of cellular transformation. HeLa, is an epithelioid adenocarcinoma of the cervix and the first human cell line to become established in culture. The parental clone, ATCC CCL2, used in our studies is the oldest available freeze-down. The isolation and initial characterization of our two HeLa revertants, HA and HF are described elsewhere (Athanassiou, M. *et al*, 1996a). Briefly,the revertant cell lines have been selected for loss of prolonged rhodamine retention by fluorescence-activated cell sorting and for morphology ; they have lost the ability to grow in suspension culture or in soft agar medium, as well as the ability to form tumors in nude mice. The HeLa cells harbor approximately 10-20 copies of the high risk HPV-18 DNA per genome equivalent (Lazo et al., 1987; Lazo et al., 1989 Schwarz et al., 1985). These sequences are preserved in the revertants and all three cell lines express comparable levels of the E7 and E6 viral oncogenes. Retroviral transduction and ectopic expression of the E6 and/or E7 in the revertants has not restored the transformed phenotype thus excluding the possibility that the revertant phenotype be an effect of an inactivating mutation p53 mRNA expression is not inhibited in HeLa cells but the levels of the p53 protein has been reported as undetectable (Matlashewski et al., 1986)or very low while p53 cDNA sequence was wild-type (Scheffner et al., 1991). p53 protein synthesis has been shown to take place by transfection of a mutant mouse p53 and detection of endogenous wild type p53 by co-immunoprecipitation of mouse-mutant/human-wild-type p53 complexes (May et al., 1991). In our parental HeLa clone as well as in the revertants p53 mRNA is wild-type in sequence and p53 mRNA levels are comparable. Molecular characterization of HA and HF has established that the reversion of transformation results from two distinct events albeit that increase of wild-type p53 protein levels appeared to be an obligatory occurence despite continued presence of the E6 oncoprotein. (Athanassiou et al; 1996a). We have hypothesized that the increase in wild-type p53 levels in the revertants as compared to HeLa may have resulted in the manifestation of wild-type p53 functions and thus have caused/contributed to the revertant phenotype of either or both revertant cell lines. In support of this hypothesis, we report in this paper that revertant but not HeLa p53 displays constitutive and inducible transcription factor properties that are thought essential for p53 function in cell growth control. Furthermore, retroviral transduction of the wild-type p53 sequences in the HeLa cells is able to suppress anchorage independent cell growth at least one hundred fold. Although we cannot yet conclude as to the extent of p53 contribution to the revertant phenotype we have identified inhibition of p53 proteolysis as a cause for the increased p53 levels in both revertants. Furthermore E6-p53 interactions necessary for the

ubiquitin-dependent degradation of p53 are interrupted in both revertants. Thus loss of E6 function is identified as the cause for differential p53 degradation and p53 activation in both revertants. We have also observed interruption of p53-p34cdc2 complex formation in the HF revertant and it is possible that lack of this interaction may be upstream of the loss of E6 function.

Materials and methods

Cell Culture:

HeLa CCL2, C4I, C4II, FS4 and Saos-2 cells were purchased from American Type Culture Collection. A431 cells were kindly provided by the Laboratory of Robert Weinberg. C4I and C4II cells were grown in Waymouth's Medium supplemented with 10% fetal bovine serum (FBS). FS4, A431, and Saos-2 cells were grown in DMEM with 10% FBS. HeLa and revertants were grown in MEMN + 10% FBS or in MEMN +10% Nu Serum (Collaborative Research). Growth in soft agar was assessed by seeding 1 X 10^2, 1 X10^3 and 1 X 10^4 cells in 2.5ml of MEMN containing 10 % FBS and 0.3% molten Difco noble agar. Plates were incubated at 37° C in a 5% CO_2 atmosphere. Plates were fed with 1.0 ml of 0.3% agar medium every 7-10 days. At the end of 3 weeks colonies were stained with 1ml tetrazolium violet overnight, then inverted and dried on 3MM Whatman paper. Colonies were counted from the plates with 10^3 cells plated.

Cells were grown to 70% confluence prior to continuous treatment with Actinomycin-D (Sigma or Boehringer Manheim) or exposure to single γ-irradiation at the doses ^{137}Cs γ-Ray Cell indicated in the figure legends. Cells were harvested at appropriate time points post-treatment as noted.

Plasmids

The pG13 and pmG15 used in chloramphenicol acetyltransferase assays have been described in Kern *et al* and were kindly provided by Dr. Bert Vogelstein. Briefly, both reporter constructs contain the CAT reporter gene under the control of basal promoters with 13 repeats of the p53 consensus binding site and 15 repeats of a mutated consensus site respectively.

To generate plasmid pSP73-p53 -B/S a 1300bp BamHI/SmaI fragment containing the entire wild-type p53 ORF DNA sequences was subcloned from pC53-SN3 (released by Dr. Bert Vogelstein, Baltimore) into the pSP73 vector (Promega). The latter plasmid was used to generate the RNA probe used in the RNAase protection assays. A plasmid from Ambion Inc., carrying a proprietary mutant p53 sequence was used to generate a positive control RNA probe.

The BamHI inserts from plasmids pC53-SN3 and pC53-175 (released by Dr. Bert Vogelstein, Baltimore) respectively contained the wild type p53 sequences and the codon 175 mutant sequences. They were subcloned into BamHI-linearized retroviral vector pLXSN and resulting plasmids pLXSN-p53 and pLXSN-175p53 were used in the retroviral transduction experiments.

Northern Blots

Cells were lysed in 1M guanidine isothiocyanate and RNA was isolated by the acid phenol method (Chomczynski and Sacchi, 1987). Aliquots of 20μg of total mRNA were electrophoresed through a 1.2% agarose gel containing 6% formaldehyde. The RNA was transfered to Hybond N membrane (Amersham) electrophoretically in 25mM phosphate buffer pH6.5, at 1Amp for 2hours using the Bio Rad transblot apparatus and fixed by ultraviolet irradiation. Blots were hybridized in 50% formamide, 1M NaCl and 1% SDS for 30min then hybridized overnight at 42°C to radiolabeled probes corresponding to either waf-1, gadd-45 or p53. Waf-1, 28SRNA and gadd-45 oligonucleotide probes were end-labeled with ^{32}P-ATP (5000ci/mmol; NEN Dupont) using polynucleotide kinase (BRL) by the random priming method. The gadd-45 probe was kindly provided by A. Fornace. The waf-1 probe was an amplified cDNA fragment. An 1800bp p53 cDNA probe was labeled by nick translation using DNAase I/Pol (NEB). Membranes were washed three times in 0.1M NaCl, 0.1% SDS at 65°C and autoradiography was performed using an intensifying screen for the indicated times at -70°C. To control for loading and blotting variations, gels were stained with ethidium bromide and photographed or membranes were probed with an oligo corresponding to 28S ribosomal RNA or with the p53 probe.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared as previously described (Dignam et al., 1983; Carrier et.al., 1992) and used fresh or frozen in N_2. DNA binding reactions were carried out for 20 min at room temperature in a buffer containing 20mM HEPES (pH 7.9), 100mM KCl, 1mM EDTA, 1mM dithiothreitol, 0.5 μg of polydI.dC, 10^4 disintegrations per minute of labeled probe, 20% glycerol and 12 μg of nuclear protein extract. The probe used was a 20mer double stranded synthetic oligonucleotide containing the consensus p53 binding sequence as reported by Funk et al. (1992). Similar results were obtained when we used a 30mer double stranded oligo corresponding to positions 1569-1598 of the human gadd45 gene (Kastan et al., 1992). Each oligo was labeled separately with T4 polymucleotide kinase (New England Biolabs) using [γ-^{32}P]ATP , 5000ci/mmol (NEN, DUPONT) before the strands were allowed to reanneal. Where indicated 0.2μg of monoclonal antibody 421 was added to the nuclear protein extract prior to its addition to the binding reaction. The samples were then analyzed on a 4% nondenaturing polyacrylamide gel.

The consensus p53 probe used was the self annealing GGA CAT GCC CGG GCA TGT CC oligonucleotide made by Ransom Hill Bioscience, Inc. Oligonucleotide was boiled then cooled down to form a double strand. Labeled probe was purified on G25 or PAGE

cDNA sequencing

poly A+ mRNa from HeLa, HAand HF was isolated using the acid extraction method of Chozminsky and Sacchi. Exons 4 to 11 were amplified by Reverse transcriptase PCR, using the Ambion p53 mutation detection Kit. Multiple clones were subcloned into the plasmid pCR specialized for the subcloning of PCR products and supplied by Stratagene. Both strands of twelve cDNA clones from each of HeLa, HA and HF were subjected to sequencing and compared to wild-type p53 sequences. No mutations were detected within the sequenced p53 region in any of the cell lines.

RNAase protection assay

An RNAase protection assay was used to compare relative levels of p53 in HeLa and revertants as well as for the detection of mutations within the p53 cDNa sequences. Plasmid pSP73-p53 -B/S was linearized with HindIII and a labeled antisense strand was synthesized from the T7 RNAse polymerase promoter this probe was hybridized to 20μg of RNA extracted by the acid guanidium extraction method (Chozminsky and Sacchi). The assay was performed using Ambion's RNAase protection kit according to the manufacturer's protocol. A second probe, synthesized from an Ambion Inc. proprietary plasmid and carrying a mutant p53 sequence was used as a positive control for mutation detection. To detect the β-actin transcripts, another probe, synthesized from HindIII- linearized pBH-11with T7 polymerase was used as an internal control. Riboprobes were labeled with [α-^{32}P]UTP at 15Ci/mmol. 10μg total RNA was hybridized with wild-type or mutant p53 probe and with the β-actin control probe in the same reaction tube after incubation at 53^{o}C for 15hr. The samples were digested with RNAase A and RNAase T1 and the protected fragments separated in a 5% denaturing polyacrylamide gel and run at 200V for 1hr. The gel was then dried and exposed to Kodak X-AR film overnight.

CAT assays

Appoximately 5 x 10^6 cells were transiently transfected by electroporation with 10μg pG13, pmG15. As a control, cells subjected to the sane pulse in the absence of DNA. Electroporation was carried out at room temperature in culture medium at 270mV/cm^2 using a Bio-Rad Gene Pulser apparatus. Twenty-four hours post-transfection, appropriate samples were treated with 0.45nM ActinomycinD for 4hrs or subjected to 600Rad of γ-irradiation. Cell lysates were prepared 48hrs post-transfection and a continuous enzymatic assay (Eastman, Biotechniques 1987) was carried out over a time course of 3hrs to quantitate chloramphenicol acetyltransferase

activity on tritiated acetyl-CoA. Linear curves of CAT activity over time were plotted and relative CAT activity rates were calculated using the Cricket Graph software. Half of each cell sample was used in a PCR based assay to normalize for transfection efficiency between samples as previously described in Bahramian and Zarbl, 1994. Results from two independent experiments were averaged and shown in Table 1.2.

Immunoaffinity purification of p53:

The p53 protein from HeLa, HA, HF, A431 and Saos-2 cells was purified on a mAb 1801 column (p53 ImmunoCruz, Santa Cruz Biotechnology) according to the supplier's protocol. Cell lysates were normalized for protein content prior to run through the column. The experiment was repeated in three independent expeiments. The quantities of protein run through the column ranged from 3-20mg in 2ml of lysate. The final eluate+Laemmli buffer volume was 600ml and 1/12 of that was used per electrophoresis run. When 20mg of lysate were used cell lysates were first concentrated on Centriprep-3 columns (Amicon) prior to passage through the immunoaffinity column. Immunoaffinity columns were also set up using 100µl agarose conjugated mAb 240 (Santa Cruz Biotechnology). 2mg of protein extract was run through the mAb 240 column using the same procedure as with the ImmunoCruz columns.

Western Blotting:

Protein samples were boiled in sample buffer for three minutes prior to loading on a Laemmli gel. SDS-PAGE was carried out in Tris-Glycine buffer system (Harlow, #715) under constant voltage of 80 V overnight using in either the Mini Protean (BIO-RAD) or a minigel apparatus from CSB Scientific Co. for 2 hr, or at 150V on a Protean Apparatus (BIO-RAD). Protein standards used were BIO-RAD low molecular weight prestained standards and BIO-RAD wide range standards, which were visualized after transfer by treatment of the Western Blot with the Aurodye Kit (Amersham) according to the manufacturer's protocol. Separated proteins were transfered overnight on a PVDF membrane (Immobilon-P; Millipore). The transfer was performed in the cold in the Trans Blotter apparatus (BIO-RAD) under constant voltage at 60 V in transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Upon completion of the transfer, membranes were blocked in Blotto (5% milk in TBS-T) for 30min, then incubated with the appropriate antibody dilution in Blotto for 1hr 15min at room temperature. Blots were then washed twice with TBS-T (pH 7.6; 20mM Tris, 137mM NaCl; 0.1% Tween 20) for 10min at room temperature, incubated with a horseradish- peroxidase-conjugated anti-mouse or anti-rabbit polyclonal secondary antibody (1:2000 dilution in Blotto; Santa Cruz Biotechnology or

Transduction Laboratories) for 30min. After being washed twice with TBS-T and once with TBS (same as TBS-T without the Tween) or PBS. Proteins on the blots were detected with the ECL reagents (Amersham) were used for antigen detection according to the manufacturer's protocol. In order to check the quality of transfer and the loading of equal amounts of protein in each lane, membranes were stripped in 0.2M ethanolamine at 82^{o}C for two hours according to the manufacturer's protocol, washed with PBS-0.4% Tween 20 and stained in 0.1% India ink in PBS-0.3%Tween 20 (Harlow book as reference).

Retroviral transduction

Briefly, ecotropic helper-free virus was generated by transient transfection of the highly transfectable BOSC cell line (60% transfection efficiency) using a highly efficient precipitation with calcium phosphate method. High titer amphotropic split helper-free retrovirus was generated by subsequent infection of the mouse ΨCRIP packaging cell line. Infectious ΨCRIP supernatant was then incubated with HeLa and revertants for 24 hours. For all infections, infected cells were selected in 500μg/ml G418. The pull of G418 resistant cells was tested for growth in soft agar and molecularly characterized by Northern and Western analyses.

Antibodies:

Anti-HPV-18/E6 antibody C1X1 and anti-HPV16/18 antibody CP15 used respectively for E6 EMSA and Western blotting were purchased from Oncogene Science and Santa Cruz Biotechnology. For the study of complex formation between p53 and other proteins we used the ImmunoCruz system from Santa Cruz Biotechnology in which the column is loaded with monoclonal antibody 1801. For Western blotting of p53 we used monoclonal antibody DO-1 from Santa Cruz Biotechnology. For p53 EMSA assays we used the monoclonals 421 and 1801 interchangeably (Oncogene Science; Ab-3 and Ab-1).

In vitro protein degradation assay

Cells were grown to 70% confluence in 150mm culture dishes. One plate per each cell line was suspended in 20mM Tris. Cl (pH 8), 100mM NaCl, 1% NP-40, 1mM DTT, 0.01% PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin, homogenized by passage through a Quiashredder (Quiagen). The homogenates were then run through a pre-equilibrated cation exchange column (S-column ; Bio-Rad) to partially purify the E6 activity or concentrated and desalted against ubiquitination assay buffer on a Centricon 10 column. Proteins bound to the S-column were eluted with 500μl of 500mM NaCl, 20mM Tris.Cl. The column eluate was then dialyzed against 500ml of ubiquitination reaction buffer (25mM Tris. Cl pH7.5, 100mM NaCl, 3mM DTT) in a Slide-alyzer (Pierce; molecular weight cut-off 10,000) for 2hrs at 4°C. ^{35}-S methionine-labeled or non-radioactive wild-type p53 were generated in a coupled transcription-translation system (SP6 TNT system; Promega) from a plasmid kindly provided by Jon Huibregtse in P. Howley' s laboratory. 2μl of the *in vitro* translated radiolabeled p53 were incubated with 25μg of the proteins eluted from the cation exchange column or 2μl of a 1:5 dilution of unlabeled p53 were incubated with

50μg of concentrated lysate in degradation reactions of total reaction volume of 40μl. Degradation reactions were carried out in ubiquitination reaction buffer with 6μg ubiquitin and 2mM ATP or 2mM ATP-γ-S. Control reactions were run in the presence or absence of rabbit reticulocyte lysate. Reactions were ran for a time course of 3hrs at 30°C. They were then stopped with 20μl of 3X Laemmli buffer from New England Biolabs and subjected to 3min of boiling. Half of each reaction was loaded on a 10% SDS-PAGE gel. For reactions containing radiolabeled p53 the gels were dried out and exposed on a phosphorimager screen overnight. Gels of reactions carried out with non-radiolabeled p53, were subjected to Western blotting with antibody D0-1. The use of Western blotting with total protein extracts increased the sensitivity of detection and allowed us the use of less p53 per reaction.

Results

p53 protein levels are increased in revertants as compared to HeLa

Previous reports found the levels of p53 protein in HeLa to be undetectable or negligible (Matlashewski et al., 1986; Scheffner et al., 1991). We have reported that with higher sensitivity Western blotting p53 is detectable in HeLa and was found to be at least five-fold increased in the HA and the HF revertants relative to HeLa (Athanassiou et al, 1996a). While the levels of p53 in all three cell lines varied depending on cell culture confluence and nutrient availability, p53 expression was consistently higher in the revertants than in HeLa.

p53 protein has been shown to be inducible in mouse fibroblast lines by treatment with ultraviolet light or UV-mimetic drugs due in large part to increased stability of the protein. p53 induction has also been shown to take place in vivo in human skin subjected to UV. Similar responses have been shown after treatment with γ-irradiation or Actinomycin D. Exposure to these inducing agents caused a temporary arrest in the G1 phase of the cell cycle expressing wild-type p53, but not in cells with mutant p53. Furthermore, in malignant cells lacking endogenous p53 the ability to arrest in G1 after γ-irradiation was partially restored when they were transfected with a construct expressing wild-type p53. Overexpression of a mutant p53 in tumor cells with a wild-type endogenous p53 abbrogated the G1 arrest (Kuebritz et al., 1992). Cells derived from patients suffering with Ataxia Telangiectasia -a disease characterized by hypersensitivity to ionizing radiation, radioresistant DNA synthesis and a markedly increased incidence of cancer- failed to induce p53 and in a manner very similar to that observed in p53 deficient cells and failed to arrest in G1 (Kastan et al., 1992). These results suggested that cell cycle control requires the uninterrupted function of p53 inducing pathways. We have thus tested the cell lines in our in vitro system formation for their ability to induce p53 protein levels after treatment with relevant reagents such as Actinomycin D and γ-irradiation. The relative ratio of p53 in revertants vs. Hela was also found to increase upon γ-irradiation and treatment with actinomycin D (Figure 2). In the revertants,the rate of p53 induction was an order of magnitude more rapid than in HeLa and resulted in ten fold higher p53 levels in the revertants (Table I). Our results are comparable to the report by Kastan et al. (1992), who observed a seven-fold induction of wild-type p53 in normal cells versus a two-fold increase in p53-induction-deficient AT cells.

These results suggested that the levels of the p53 protein in the revertants have been restored to levels comparable to those of normal cells. Furthermore the p53 inducing pathways essential to the function of p53 in cell cycle control are again functional in the revertants. We were thus led to the hypothesis that elevated wild-type p53 expression could contribute or even

lead to the suppression of HeLa transformation in a manner analogous to data obtained for colon carcinoma, osteosarcoma and breast carcinoma cell lines (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990). We have thus undertaken to investigate whether or not and to what extent p53 function contributes to the HA and HF revertant phenotypes.

The p53 gene has not been mutated in the revertants

Previous analysis of the p53 cDNA from the HeLa ATCC CCL2 clone showed that the mRNA expressed in these cells encodes wild-type p53 (Scheffner et al., 1991). Both immunoprecipitation and combination of immunoprecipitation and immunoblotting studies have shown that the p53 protein in these cells was much lower when compared to protein in other cell lines with comparable mRNA expression levels (Matlashewski et al., 1986; Scheffner et al., 1991). Mutations within a p53 allele have been shown to increase the p53 half-life and may therefore account for increased levels of p53 protein in the revertants. It is conceivable that revertants harbored p53 mutations that resulted in stabilization of p53, while maintaining some of its tumor suppressing properties. However, all of the mutations identified to date that affect p53 stability also so far identified p53 mutations lead to loss of growth-suppressor or gain of growth-promoting function (Bargonetti et al., 1991; Kern et al., 1991; Kern et al., 1991; Zambetti et al., 1991; Bargonetti et al., 1992; Bargonetti et al., 1992; Farmer et al., 1992; Zambetti et al., 1992; Zambetti et al., 1993; Olson et al., 1994). Nonetheless, there remained a possibility that a mutation occured in the p53 gene and that the mutation prevented degradation without affecting function.

Therefore, we have employed RNAase protection assay and direct sequencing of cDNA to test whether HeLa and either or both of our revertants have suffered a mutation within the p53 gene. Hybridization of endogenous mRNA from all three cell lines with probes scanning the entire p53 ORF protected the entire probe from digestion with RNAase A and T1, while hybridization of the same probe with *in vitro* transcribed mutant p53 mRNA resulted in cleavage of the mismatch hybrid species and generated two bands on denaturing PAGE. Although this approach is fast and less laborious than direct cDNA sequencing, it has the disadvantage that it can only detect one third of point mutations due to the inability of the RNAase to detect mismatches. We have thus sequenced revertant and HeLa p53 coding sequences that correspond to the regulatory and DNA binding regions of the p53 protein, specifically exons 4 to 11. Exons 5 to 8 have been shown to contain more than 90% of the mutations observed in human tumors. We have detected no mutations within the sequenced regions in any of our three cell lines of study.

The detection of p53 in many studies has relied on the use of a panel of monclonal antibodies that recognize different epitopes of the murine and human p53 proteins (Harlow et al., 1981; Yewdell et al., 1986; Gannon et al. 1990, Cook and Lane, 1990). The wild-type p53 protein can assume two conformations during the progression of the cell cycle, where each of which may play a distinct role in the physiological life of the cell (Ullrich, et al., 1992). One conformation is specific to the wild-type protein and has been associated with the ability to block cell cycle progression. The other conformation seems to function in promoting cell proliferation and was originally identified as a characteristic distinguishing mutant p53 from wild-type. The mutant latter conformation is recognized by antibody 240. As further evidence that no mutation has occured within the p53 gene in our HeLa and revertants we attempted to purify cellular p53 from the three cell lines on immunoaffinity columns loaded with monoclonal antibody 240 that recognizes mutant p53. No mutant p53-specific signal was detected on immunoblots of the eluates from these columns (data not shown), consistent with the absence of mutant p53 in the revertants.

p53 specific endogenous transactivating activity is detectable in the revertants and inducible by Actinomycin D and γ-irradiation.

p53 was found to transactivate mammalian genes such as the muscle creatine kinase (Weintraub et al., 1993).and subsequently shown to bind DNA both specifically (Kern et al., 1991; Bargonetti et al., 1991) and non-specifically (Steinmeyer et al., 1988; Kern et al., *b*, 1991). When the p53 response element was included in basal promoters and placed 5' of the CAT reporter gene, the reporter was transactivated when co-transfected with wild-type p53 (Zambetti et al,1992; Kern et al., 1992; Farmer et al., 1992). Furthermore when mutant and wild-type p53 were co-expressed there was a dominant effect of the mutant p53 that was related to decreased binding of presumed wild-type mutant oligomers to DNA (Kern et al. 1992). The pG13-CAT and pMG-15 CAT reporter constructs have been previously described (Kern et al., 1992). They contain the polyoma virus early promoter and the CAT respectively downstream of 13 wild-type and 15 mutated repeats of a p53 binding sequence.

Differential activation of the p53 reporter construct pG13CAT without and upon transient treatment with Actinomycin D or exposure to γ-irradiation was assessed in Hela and revertants. Transfection of cells was performed by electroporation, Cells were harvested after 24 post transfection and CAT assays were carried out as described in Neumann (1987) with a modification by Eastman (1987). The technique is a continuous assay and thus yields quantitative kinetic data that can be analyzed by standard mathematical means. Data from the CAT assay were normalized for the relative transfection efficiency of the cell lines as described in Bahramian and

Zarbl (1994). Briefly, we employed a PCR based technique to directly quantitate the relative amount of transfected DNA within the nucleus rather than using a second reporter gene to normalize CAT activities. We thus eliminated variability across cell lines in the control of expression of the second reporter gene. The results of three experiments are summarized in Table 2.2. Activities reflect the rate of conversion of chloramphenicol to an acetylated form, expressed relative to the corresponding rate in the untreated HeLa cells. Activities of the control pMG15 were below the level of detection in all three cell lines with or without treatment. Thus, any differences observed in transactivation of pG13 across cell lines are due to the presence of the p53 binding sequences rather than of any element in the polyoma early promoter. That the levels of p53 transactivational activity were sufficient to detect in uninduced cells that naturally expressed p53 was surprising since in most previous work, the CAT reporrter constructs were co-transfected with wild-type p53 expresssion vectors. Transient transfections would yield much higher levels of the exogenous p53 than expected to be endogenous in a cell. Among untreated cells HF showed the highest levels of p53 transactivating activity with a relative CAT rate of 30. The activity in this cell line, was not significantly increased by treatment with Actinomycin D and was only slightly increased by γ-irradiation. CAT activity in untreated HA was twenty-fold higher than in HeLa. Transient treatment with Actinomycin D and exposure to γ-irradiation both increased the relative CAT rates two-fold compared to untreated HA thus up to fifty-fold with respect to HeLa. These results clearly display that p53 specific transactivational activity constitutively elevated in revertant cells but not in HeLa , consistent with our hypothezis that the p53 protein in the revertants is not only increased as compared to HeLa but also functional. The levels of p53 specific transctivational activity are constitutively high in uninduced cells. The observed increase in p53 function after γ-irradiation or ActD (2-fold) was not as dramatic as the observed (10-fold) increase in protein levels. These results are however in agreement with the findings of Zhan et al. (1993) who did not observe significant increases of pG13-CAT transactivation after transient transfection and treatment with 5Gy of ionizing radiation of RKO cells which carry wild-type p53 that is able to induce and G1 that is able to arrest following ionizing radiation. Therefore, our results indicate that the revertants express elevated levels of wild-type p53.

p53-specific DNA binding is inducible in the revertants but not in HeLa - EMSA

As a further test of the gain of function of endogenous p53 in revertants as compared to HeLa we carried out gel mobility shift assays using the consensus binding sequence or a sequence from the 3d intron of the gadd 45 gene (Figure 2.3). Upon incubation of p53 specific antibody with the labed double stranded oligonucleotide and the nuclear extracts, a distinct band with a shifted mobility was seen in treated revertants and positive control FS4 cell extract. The shifted band was not visible in the absence of incubation with the 421 antibody in induced revertant cell extracts and was blocked with addition of excess 100-times excess of unlabeled oligomer. Nuclear extracts were tested for the presence and induction of p53 by Western blotting. These results demonstrate that an inducible factor that contains p53 can bind p53 binding sites in the revertants but not in HeLa cells.

Constitutive expression of gadd45, inducible expression of waf-1 in the revertants but not HeLa

Damage to DNA in both prokaryotic and eukaryotic cells lead to transient delays of cell cycle progression by blocking the passage from the G1 phase to S and from the G2 phase to M which presumably respectively prevent replication of damaged DNA and segregation of damaged chromosomes. Until p53 was implicated in G1 arrest following γ-irradiation (Kastan et al., 1991) little was known about the mechanism that led to the cell cycle arrest following DNA damage. Kastan et al., (1992) showed that in Ataxia Telangiectasia cells which failed to arrest in G1 much like in p53 defficient cells, p53 protein induction failed following γ-irradiation as did the induction of the mRNA of the growth arrest and DNA damage inducible gene gadd-45. The same authors correlated the lack of gadd45-mRNA induction with lack of p53 binding to a binding site within intron 3 of the gadd45 gene. Zhan et al. (1993) provided further evidence suggesting that p53 involvement in gadd45 induction is specific to γ-irradiation and not other treatments. We have therefore tested the state of the gadd45 mRNA in our HeLa and HeLa revertants by Northern hybridization. The results (Figure 2.4). Demonstrated that the levels of Gadd45 mRNA were at least 10-fold lower in HeLa than in revertants, without prior treatment of cells with γ-irradiation. Although we did not find the gadd45 mRNA levels in the revertants to be induced by γ-irradiation ,the fold difference in HeLa and revertant gadd45 mRNA is equal to the maximal induction reported for any cell line (RKO cells; Kastan et a., 1992). Given that HA and HF are both clones of HeLa we could consider gadd45 levels already maximally induced with respect to HeLa gadd45 mRNA levels. Furthermore, the fact that gadd45 expression is constitutive is in agreement with the CAT assay data that detect p53 specific transactivating activity even in

unstimulated cells. Finally, p53 specific binding of the gadd45 binding site is detectable in the revertants but not in HeLa and appears inducible in at least the HA revertant. It remains possible, however, that despite a functional p53 in the revertants, the γ-irradiation-dependent pathway of gadd45 induction may be interrupted by mutation of some factor necessary for p53 mediated gaddd45 DNA binding and gaddd45 mRNA induction. It is conceivable that such a factor may have been mutated during the trasformation of the HeLa cell.

The WAF-1 gene was first identified by its differential induction by wild-type p53, but not mutant p53. The induction is thought to be mediated by direct transactivation of the waf-1 promoter by p53, since the identified p53 binding sites within this promoter can confer p53 specificity to reporter constructs (El Deiry et al., 1993). WAF-1 was then found to be identical to a gene called CIP-1 which encoded an inhibitor of cyclin/cyclin kinase complexes (Harper et al., 1993). A connection was thus established between p53 and cell cycle control, and evidence has since been accumulating to support the hypothesis that induction of p53 under conditions of cellular stress or DNA damage leads to inhibitiion of cell cycle progression through the waf-1/cip-1 protein. All current evidence suggests that wild-type p53 function is absolutely essential to waf-1 induction (El Deiry et al., 1993). As a further test for the restoration of wild-type p53 function in our HeLa revertants, we have assessed the state and inducibility of waf1 mRNA. The indicated that waf-1 mRNA is inducible by γ–irradiation in the revertants, but not at all in HeLa cells.

Taken together results results from CAT assays, DNA binding and expression of downstream p53 effectors established that both revertants not only show increased levels of p53, but have gained wild-type p53 function. Therefore, we have detected a very tight correlation between activation of p53 function and suppression of the malignant phenotype in 2 independent revertants of HeLa cells.

Wild-type p53 can suppress HeLa transformation

Having thus established that wild-type p53 function had been restored to the revertant clones during the reversion process, we wished to determine whether wild-type p53 function alone was sufficient to suppress the HeLa transformation. p53 inactivation by E6 is thought to be an initiating step of cervical carcinogenesis and hence of Hela transformation. Although inactivation of p53 is thought to contribute to the genomic instability that is further thought to promote oncogenic/transforming mutations required for the malignancy of tumors, re-introduction of p53 suffices to supress the transformed phenotype in many tumors, independent of the presence of such secondary mutations. Moreover, previous results have shown that

decreasing E6 activity by Antisense RNA can inhibit transformation of HeLa, presumably by increasing p53. Therefore, we expected that reinroduction of p53 in the HeLa cells would suppress transformation, provided that exogenous p53 would exceed the saturation point of the E6 pathway that targets p53 for degradation in the HeLa cells. We have employed retroviral transduction to introduce p53 in the HeLa cells. The retroviral vector, LXSN, into which the wild-type p53 sequences were cloned, is a widely acceptable vector that also drives the expression of the neomycin resistance gene in infected cells. Thus succesfully infected cells gave rise to G418 resistant clones that expressed exogenous p53, as verified by Northern blotting in which it was possible to distinguish the exogenous RNA of 1800b from the 2400b -long endogenous HeLa mRNA (data not shown). The pool of Hela cells expressing exogenous p53 (HeLa-p53) was compared to HeLa by soft agar growth assay. Clonigenicity in soft agar was thus shown to be reduced 100-fold by introduction of exogenous p53 into the HeLa cells. Western blotting analysis showed that the levels of p53 protein in Hela-p53 are not as high as revertant p53 levels. This finding was consistent with the obsevation that growth of the revertants in soft agar is even more reduced when compared to HeLa-p53 pool. Our previous results demonstrated retroviral transduction of E6 does not have an effect on the revertant phenotype, suggesting that the E6 functional pathway is interrupted/inhibited in the revertants. That would not be the case for Hela p53 where endogenous E6 would still antagonize exogenous p53 expression. In any case, retroviral transduction of p53 into HeLa suppressed transformation to a significant extend and suggested that gain of p53 function could suffice to revert Hela cells from tumorigenicity.

Disruption of p53 Function in HeLa Revertants.

We so attempted to interrupt wild-type p53 function in the revertants through retroviral transduction in the revertants of an exogenous dominant mutant p53, the His to Arg 175 mutant. Previous work has shown that p53 is functional in tetrameric complexes (Friedman et al., 1993). As previously mentioned, wild-type p53 can exist in different conformations, one of which is unique to the wild-type protein and termed the wild-type conformation while mutant p53 is "trapped" in the so-called mutant conformation. Milner and coworkers have demonstrated that when mutant and wild-type p53 are co-expressed *in vitro* (1:1 molecular ratio) the wild-type p53 polypeptides were forced into the mutant conformation. Furthermore Kern et al. showed that transiently transfected 175 mutant could reduce the p53 transactivation of reporter constructs inthe presence of cotransfected wild-type p53. We reasoned that if the same effect would occur *in vivo,* then ectopic expression of the mutant p53 would drive the formation of inactive dimers and tetramers with endogenous wild-type p53. This would decrease wild-type p53. Thus,

expression of a dominant negative mutant in a revertant that has been generated through p53 activation would conceivably retransform this revertant. However, retroviral transduction of the 175 mutant did not result to increased clonigenicity of the reveratnts in soft agar although we did observe a slight increase in the size of soft agar colonies in the HA cell line expressing the mutant p53.

In retrospect, the failure of dominant negative mutant was expected if endogenous p53 in the revertants had gained wild-type p53 function. First, suppression of anchorage independent growth in soft agar or of tumorigenicity in nude mice by ectopic expression of wild-type p53 occur in the tumors examined irrespective of whether they tumors express mutant p53. Second, no tumor has been described that carries simultaneously a mutant and a functional wild-type p53 gene. Third, Ullrich et al., showed that in a human glioblastoma cell line containing endogenous mutant p53 ectopic expression of inducible wild-type p53 gene, both wild-type/wild-type and wild-type mutant complexes exist. In these cells, the wild-type p53 can exert its antiproliferative effect and upon G1 arrest there are no wild-type/mutant complexes in the cell. Thus, the allosteric effect of mutant p53 may not occur *in vivo* in the same dirrect manner as *in vitro.* Finally, in Saos-2 cells which lack endogenous p53, simultaneous retroviral transfer and expression of the wt and a dominant mutant p53 genes was able to suppress anchorage independent growth and tumorigenicity much as expression of wild-type alone (Chen et al., 1990). Similarly, Wang et al., (1993) have reported that retroviral gene transfer can suppress the growth of MDA-MB468 a breast carcinoma cell line overexpressing mutant p53. Thus, in the *in vivo* contexts examined, wild-type p53 has a dominant effect over the mutants biochemically defined as dominant. Results from the retroviral transfer and expression of the 175 mutant in the revertants taken together with the manifestation of endogenous p53 function in the revertants are therefore consistent with a dominance of wild-type p53 function *in vivo* in the context of the revertant cells.

Inhibition of p53 Degradation in the Revertants

In order to define the mechanism underlying increasing p53 in revertants, we first considered E6 function, since functional inactivation of E6 in either revertant would ultimately lead to lack/reduction of p53 ubiquitination and degradation. It was also conceivable that interruption of p53 ubiquitination or degradation could occur independent of E6 function. We have thus performed *in vitro* experiments after Scheffner *et al.* (Scheffner et al., 1990) to test whether *in vitro* translated p53 is differentially degraded in HeLa and revertant lysates. Figure 2.5 shows the results of two independent experiments, the first one of which was carried out after partial purification of basic proteins to concentrate HeLa and revertant E6 protein. The remaining

components of the ubiquitin-dependent degradation pathway, including the ubiquitin ligase E6-AP are provided from the rabbit reticulocyte lysate added in the degradation reactions. The results of this experiment showed that p53 degradation is faster in the presence of a basic protein function from Hela than from revertants, thus suggesting inactivation of E6 mediated proteolysis of p53 in the revertants. The degradation of ubiquitinated p53, but not the ubiquitination itself, can be blocked by ATP-γ–S. Thus addition of ATP-γ–S to the degradation reaction mixtures would allow the formation and recognition of high molecular weight ubiquitinated intermediates. In cellular extracts of all three cell lines, HeLa, HA and HF, ATP-γ–S inhibited p53 proteolysis to the same extent demonstrating that the differential degradation of p53 in the revertants is due to aberration of the ubiquitin dependent pathway rather than a general proteolytic pathway. Furthermore, after addition of ATP-γ-S, bands of higher molecular weight than p53 but lower than would be expected for fully multi-ubiquitinated species are seen in revertant sample lanes. The second experiment presented in Figure 2 5 was carried out using total cell lysate in the degradation reactions. In order to increased the specificity of detection, in this second experiment we decreased the amount of *in vitro* translated p53 in each degradation reaction by four-fold and used Western blotting to visualize the fraction of p53 protein remaining upon a 3hr incubation with cellular extracts. As in the previous experiment degradation reactions contained reticulocyte lysate to enrich for the components downstream of p53-E6 binding. Under the conditions described and in the absence of ATP-γ-S, significant proteolytic degradation of p53 was observed in HeLa , HA and control reactions with reticulocyte lysate. p53 proteolysis has been shown to take place in reticulocyte lysates in the absence of E6 (Ciechanover et al., 1994) and the vast excess of the reticulocyte lysate proteolytic components may be masking the differences between HeLa and the HA revertant. On the other hand, degradation of p53 in the HF revertant lysates appeared to be altogether inhibited suggesting that an inhibitor of p53 proteolysis is active in this revertant. Again a band of higher molecular weight than the *in vitro* translated p53 was specifically seen in reactions containing HF revertant lysates even in the absence of ATP-γ-S and in both revertant lysates in the presence of ATP-γ-S. We shall call this band, band A. Direct immunoblotting of samples from both experiments described in this section, with anti-ubiquitin antibodies recognized a band that could be superimiposed with band A from identical gel runs. However, since reticulocyte lysate were included in the degradation reactions, a multitude of ubiquitinated species were recognized by the anti-ubiquitin antibody. The resulting high background in the Western detection has thus precluded the identification of any ubiquitinated species as conjugates of p53 without prior immunoaffinity purification of p53 species.

Taken together, results from both experiments suggest that differential degradation of p53 may be the cause of p53 accumulation in both revertants. Our data suggests the presence of an inhibitor of p53 degradation in the HF revertant. Future experiments will dissect in greater detail

the ubiquitination-degradation pathways in HeLa and revertants and will aim at identifying the cause of differential p53 degradation in these three cell lines.

Interruption of E6-p53 binding in the revertants

A combination of immunoaffinity purification of p53 and complexed proteins and immunoblotting was used as a very sensitive assay that enabled us to study the association of p53 and E6. We found that E6-p53 binding has been disrupted in the revertants. Such disruption could account for the increased levels and gain of function of p53 in HA and HF and could have resulted: i) from changes in the post-translational modification of E6 or p53; ii) from abberant expression of E6-AP, the 100KDa protein that participates in a quaternaty complex formation with p53, induces E6-p53 binding and functions as a ubiquitin ligase (Huibregtse et al., 1991; Scheffner et al, 1993); iii) from mutation of the p53 gene (Scheffneret al., 1992). These results provide veidence for the interruption of E6 function in the revertants andcan explain the previous finding about differential degradation of p53 in HeLa and revertants as well as the increase in p53 protein levels in the revertants.

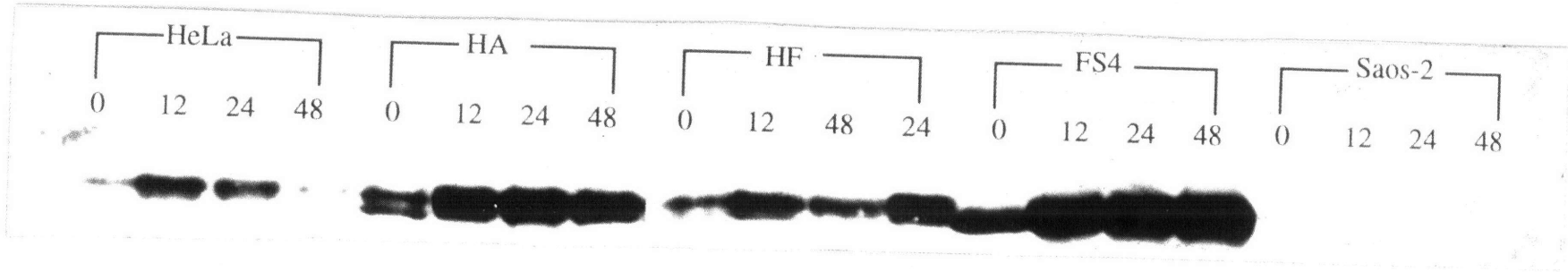

HeLa
0 12 24 48
HA
0 12 24 48
HF
0 12 48 24
FS4
0 12 24 48
Saos-2
0 12 24 48

Figure 2.1: Differential p53 induction by ActD between HeLa and revertants.

Western blot of p53 using monoclonal antibody DO-1, on 50μg of total cell lysate of HeLa, HA, HF and control cell lines. Cells were induced with 0.045nM of Actinomycin D for 0, 12, 24 and 48hrs prior to cell lysis. Cell lysis was carried out in boiling lysis buffer (10mM Tris, 1% SDS) and cell lysate was homogenized by passage through a 23 gauge needle. Protein quantitation by the Pierce BCA kit was carried out prior to addition of 2X Laemmli sample buffer. 50μg of protein were loaded per lane of a 20cm X 20 cm 8% Laemmli SDS-PA gel. Gel Electrophoresis was carried out under constant current of 0.35Amp, at 4°C, for 12hr. Relative p53 levels were quantitated by scanning laser densitometry using an LKB Laser densitometer.

Table 2. I: Kinetics of p53 induction in response to Actinomycin D in HeLa and revertants

Cell Line	Rate of p53 induction	Relative Rate	Curve Fit (R^2)
HeLa	0.083	1	0.254
HA	1.542	18	0.998
HF	1.053	13	0.953
FS4	5.500	66	1.000

Table 2.I: Relative levels of p53 protein as determined by Western blotting using monoclonal antibody DO-1 according to the protocol described in Figure 1. Cells were induced with 0.45nM of Actinomycin D for 0, 12, 24 and 48hrs prior to cell lysis. 50mg of protein were loaded per lane of a 20cm X 20 cm 8% Laemmli SDS-PA gel. p53 levels were quantitated by scanning laser densitometry of linear films exposures using an LKB Laser densitometer as described in the legend of Table II. No p53 was detected in p53 negative control cells Saos-2. Curves of p53 induction, rates and curve fits were obtained using the program Cricket Graph,. Relative Rates were obtained with reference to the HeLa cell line. p53 is induced by ActD in revertants at a 10- to 20- fold higher rate than in HeLa. p53-mediated response to ActD treatment is thus faster and yields higher levels of p53 protein in the revertants than in HeLa cells.

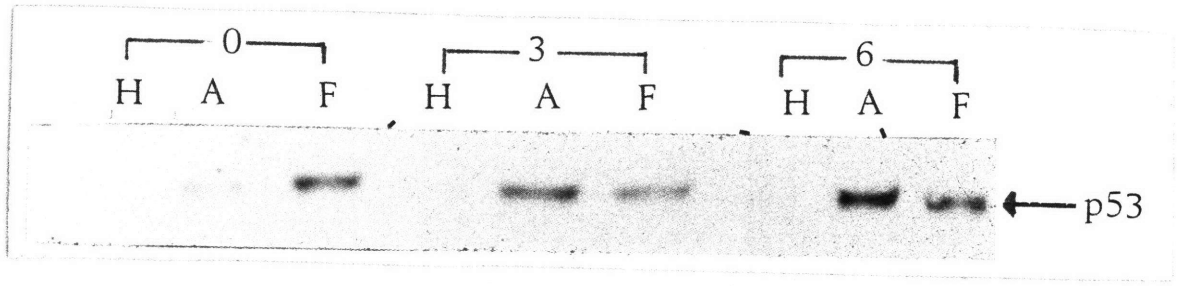

0
3
6
H A F
H A F
H A F
p53

Figure 2.2: Induction of p53 by γ-irradiation in HeLa and revertants.

Western blot on 25μg of total protein from cells dosed with 0, 3 or 6 Gray of γ-irradiation four hours prior to preparation of cell extracts. The primary antibody was horseradish peroxidase-conjugated DO-1 (Santa Cruz Biotechnology). Cell lysis was carried out in boiling lysis buffer (10mM Tris, 1% SDS) and cell lysate was homogenized by passage through a 23 gauge needle. Protein quantitation by the Bio-Rad AB kit was carried out prior to addition of 2X Laemmli sample buffer. A 10% Laemmli SDS-PA mini-gel was run. Relative p53 levels were quantitated by scanning laser densitometry using an LKB Laser densitometer. H = Hela, A = HA, F = HF.

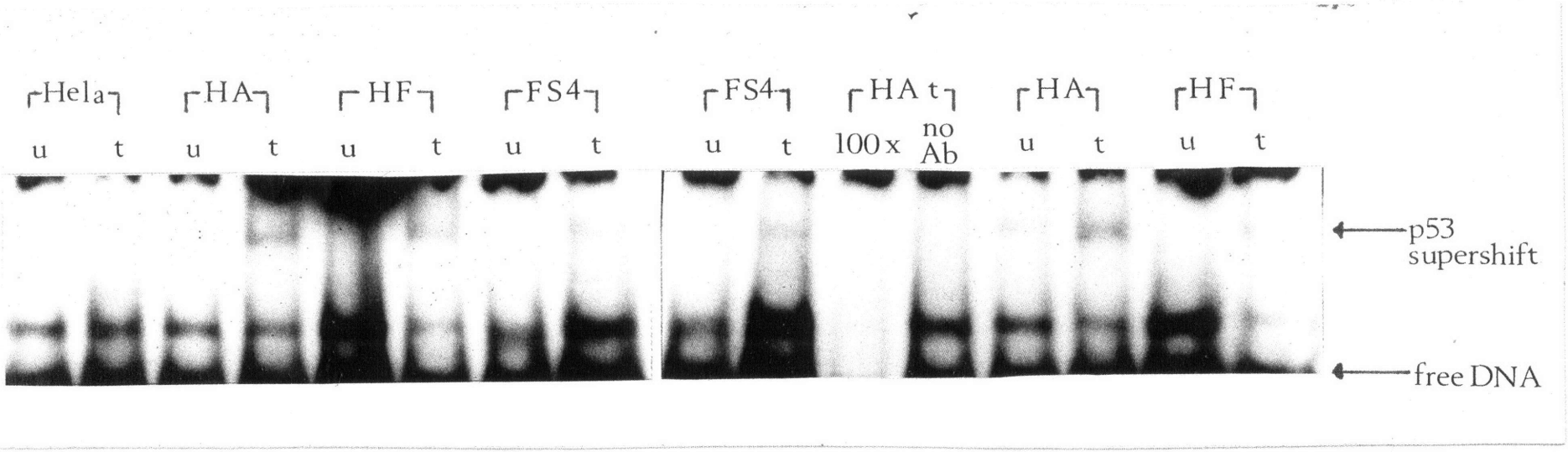
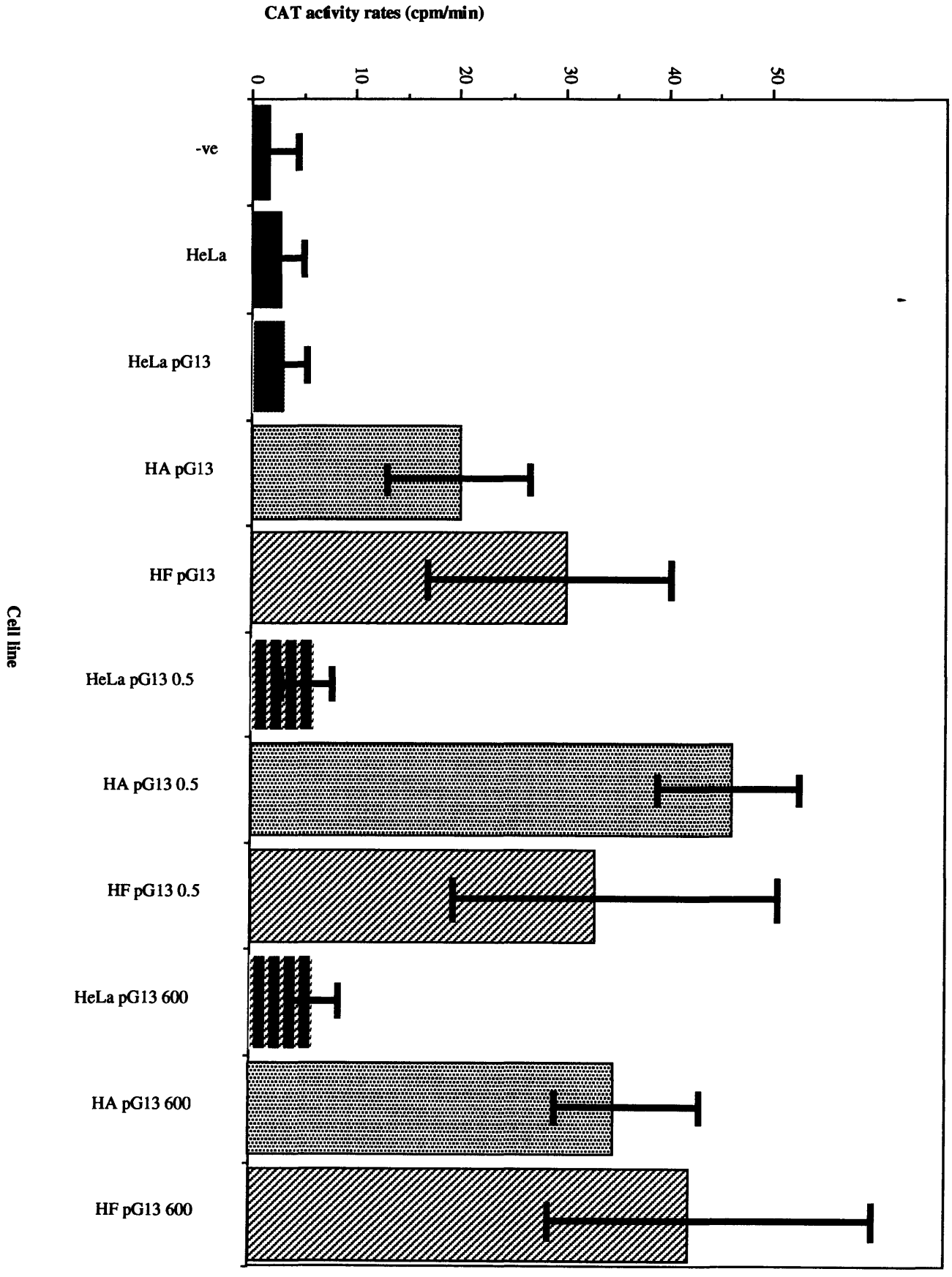
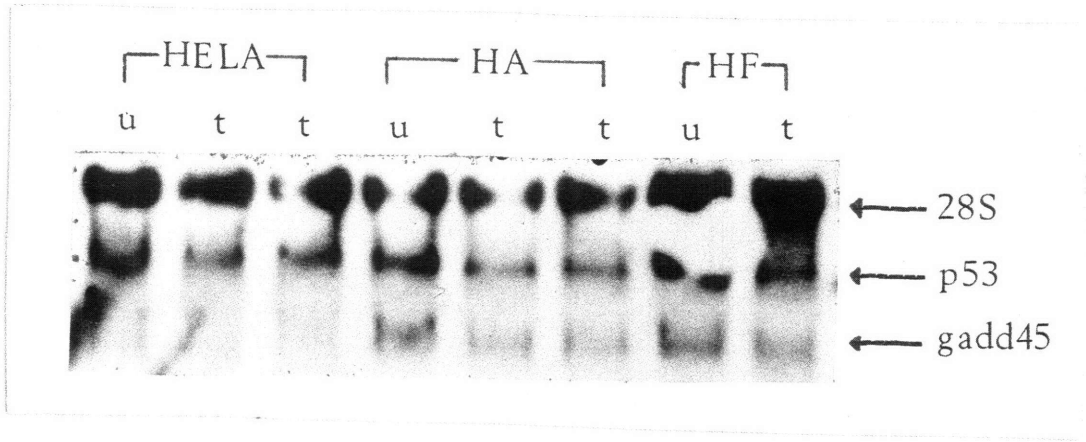

Hela
HA
HF
FS4
FS4
HA t
HA
HF
u
t
100 x
no Ab
p53 supershift
free DNA

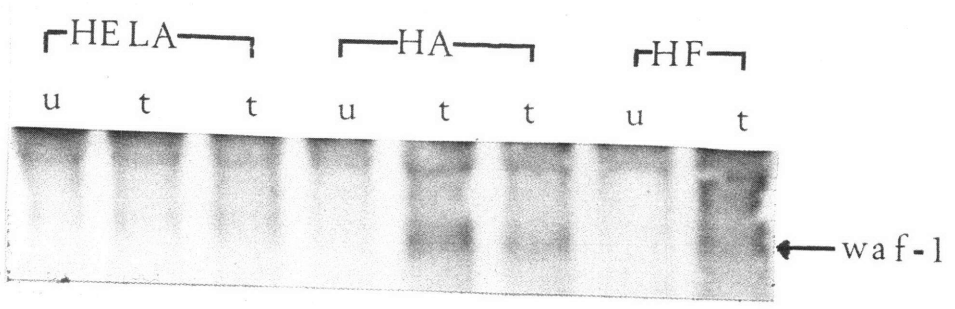
Figure 2.3: Differential DNA binding by p53 between HeLa and revertants.

Cells were treated with 0.45nM Actinomycin D for 4hrs prior to preparation of nuclear extracts as previously described (REF). Electrophoretic mobility shift assays were carried out with a ^{32}P-end labeled oligonucleotide that contained a single repeat of the consensus p53 binding site as previously described (REF). Induction of p53 was verified by Western blotting on 15μg of nuclear extract. p53 bound oligo was supershifted by the monoclonal antibody 421 (Oncogene Science; Ab-1). 100x=competition with 100-times molar excess of unlabeled oligo, u = untreated cell extract, t = cells treated with Actinomycin D, no Ab = no antibody. FS4 are positive control normal fibroblast cells. Similar results were obtained with an oligonucleotide from the gadd45 gene (Kastan et al. 1992). Induction of p53 protein by actinomycin D treatment is followed by increased binding of p53 to its consensus DNA sequence in the revertants but not in Hela. Binding to the consensus sequence is characteristic of wild-type p53. The data thus supports the idea that p53 in the revertants has maintained wild-type properties. Differential DNA binding by p53 between HeLa and revertants further supports the idea that p53 has been functionally activated in the revertants.


CAT activity rates (cpm/min)
0
10
20
30
40
50
-ve
HeLa
HeLa pG13
HA pG13
HF pG13
HeLa pG13 0.5
HA pG13 0.5
HF pG13 0.5
HeLa pG13 600
HA pG13 600
HF pG13 600
Cell line

Graph 1: $5*10^6$ cells were transiently transfected with 10μg of CAT-reporter constructs pG13-CAT or pmG15-CAT. pG13-CAT and pmG15-CAT correspondingly carry the CAT gene under the control of polyoma late promoter preceded by 13 repeats of wild-type p53 consensus binding sites and 15 repeats of mutated p53 consensus binding sites. This experiment showed the manifestation of wild-type-p53 specific transactivation in the revertants as opposed to HeLa . Treatment with Actinomycin D and γ-irradiation which induce p53 protein also induce p53-specific transactivation.


HELA
HA
HF
u t t u t t u t
28S
p53
gadd45


HELA
HA
HF
u t t u t t u t
waf-1

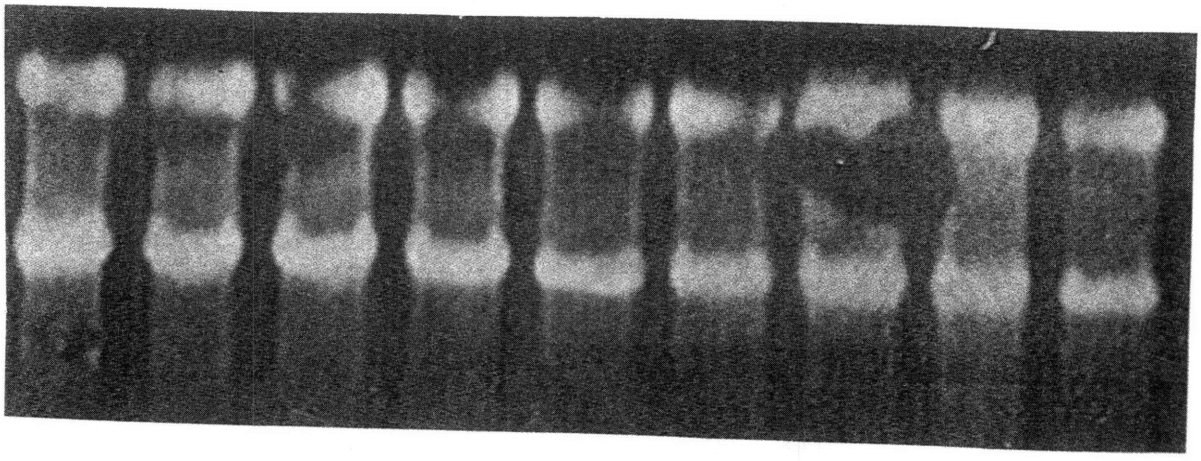


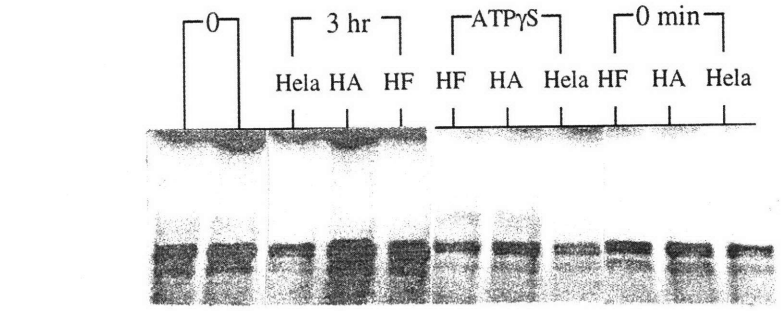
Figure 2.4: Differential expression of gadd45 and waf-1 in HeLa and revertants.

Northern blot on 15μg of total mRNA from HeLa, HA and HF exposed to 5 Gray (t for treated) or 0 Gray (u for untreated) of γ-irradiation. Top panel: Gadd 45 is expressed in the untreated revertants but is not detectable in treated or untreated Hela cells. Middle panel: Waf-1 is induced by γ-irradiation in the revertants but not in HeLa. Bottom panel: Ethidium bromide staining of the Northern blot to ensure equal loading and transfer.

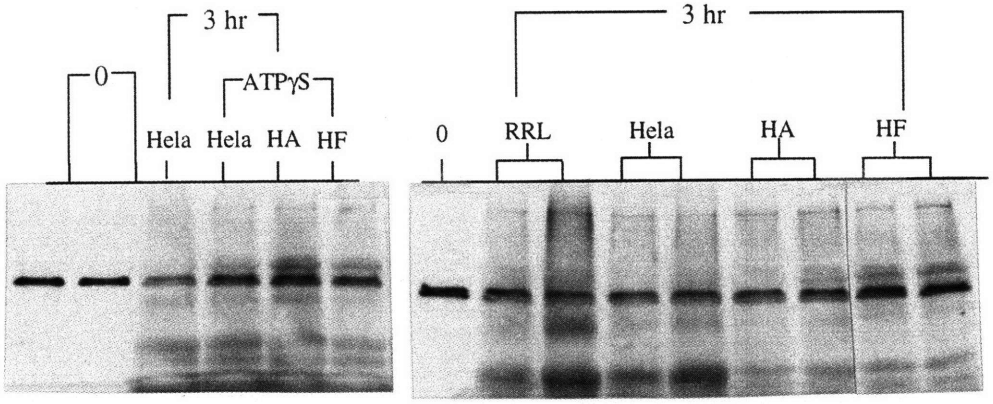
Table 2.III: Retroviral transfer and expression of wild-type p53 suppresses Hela transformation

Cell Line	Cloning efficiency (%)
HeLa + LXSN	40
HeLa + wt p53	< 0.4
HA + LXSN	< 3
HA +mut 175	< 2
HF + LXSN	< 0.1
HF + mut 175	< 0.1

Table 2.III: Exponentially growing HeLa carrying the wild-type p53 or vector LXSN alone were removed from dishes by trypsinization and diluted with MEMN + 10% FBS. Aliquots containing 10^4 cells and 10^3 cells were removed and seeded into molten Agar in 30mm dishes. Results shown here represent averages of two independent experiments carried out in duplicates showing the difference in anchorage independent growth per 10^3 cells seeded between HeLa expressing p53 and the corresponding control Hela LXSN cells. Also shown are the differences per 10^4 cells seeded between revertants expressing mutant175 (His to Arg) p53 and the corresponding control cells with LXSN.


0
3 hr
ATPγS
0 min
Hela HA HF HF HA Hela HF HA Hela

(a)


3 hr
0
ATPγS
Hela Hela HA HF
3 hr
0 RRL Hela HA HF

(b)

Figure 2.5: Differential p53 degradation in HeLa and revertant lysates.

(a) Autoradiogram of p53 degradation reactions with partially purified E6 activity from HeLa and revertants. Degradation reactions were carried out in ubiquitination reaction buffer with 6μg ubiquitin and 2mM ATP or 2mM ATP-γ-S. Reactions were ran for a time course of 3hrs at 30°C. They were then stopped with Laemmli bufferand half of each reaction was loaded on a 10% SDS-PAGE gel. Gels were dried out and exposed on a phosphorimager screen overnight.
(b) Western blot of of p53 degradation reactions with total cell lysates. Gels of reactions carried out with non-radiolabeled p53, were subjected to Western blotting with antibody D0-1.

Both (a) and (b) H= HeLa, A = HA, F = HF. 0 time pont contained no extract and was kept on ice for 3hours. Samples containing ATP-γ–S as indicated. RRL = Reticulocyte Lysate Extract only included in the degradation reaction. * = band A (see Results section).

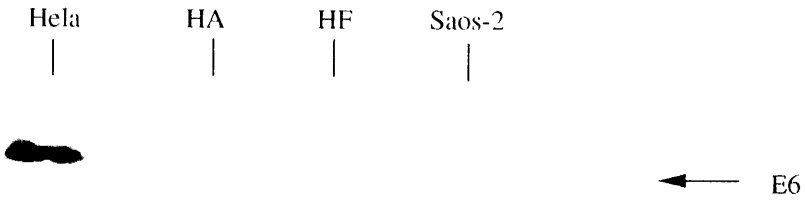

Hela
HA
HF
Saos-2
E6

Figure 2.6: E6 binding to p53 is interrupted in both revertants

Western blot of E6 following a immunoaffinity purification of p53 and associated proteins. A 15% mini Laemmli SDS-PA gel was run for 2hrs at 80V.

Discussion

In order to study cervical cell transformation we have employed an *in vitro* cell culture system comprising of the cervical carcinoma cell line HeLa and two non-tumorigenic variants (revertants), HA and HF. Such systems have been used succesfully to identify the ras tumor suppressor gene krev (Noda et al., 1991) and transformation effector genes (Boylan et al., 1991; Kho et al., 1991), whose products comprise and/or regulate the biochemical pathways of cellular transformation. The isolation and initial characterization of our two HeLa revertants, HA and HF are described elsewhere (Athanassiou, M. *et al*, 1996). Briefly,the revertant cell lines have been selected for morphology and for loss of prolonged rhodamine retention by fluorescence-activated cell sorting; they have lost the ability to grow in suspension culture or in soft agar medium, as well as the ability to form tumors in nude mice. The HeLa cells harbor approximately 10-20 copies of the high risk HPV-18 DNA per genome equivalent (Lazo et al., 1987; Lazo et al., 1989 ; Schwarza et al., 1985). These sequences are preserved in the revertants and all three cell lines express the E7 and E6 viral oncogenes. Retroviral trasnduction of E6 and E7 or hybrid E7/E7 mRNA in the revertants had no effect in the revertant phenotype. Since maintained function of the revertants has been shown to be necessary for the mmaintenance of the transformed phenotype, one possible explanation for the latter result would be that the E6 and/or E7 functional pathways have been interrupted in the revertants. The transforming functions of E6 and E7 culminate in their respective binding of the p53 and Rb tumor suppressor genes. To assess E6 and E7 function we have examined the state of the Rb and p53 in Hela revertants. In both revertants and particularly HF, we found Rb-E2FI interactions which were non-detectable in HeLa. But the most striking similarity between the two revertants was an increase in p53 protein level with respect to HeLa.

The p53 in our parental HeLa clone has been reported to be wild-type (Scheffner et al., 199). Mutation of the p53 gene could have increased p53 protein half-life in the revertants and could have resulted in the observed increase in p53 protein levels in the revertants. HA and HF, however, are independent revertants as shown by their distinct growth and morphologies, by a difference in their hybridization pattern with a human VNTR probe as well as by the differential expression of a number of genes tested. Since there are about one million genes within the human genome, the probability of mutation within one given gene is 10^{-6}. Thus the probability of mutation within the p53 in two independent revertants is about 10^{-12}. Moreover, p53 mutation would be likely to negate the reversion process thus its occurence in both independent revertants would be counterintuitive. Nevertheless we have tested for p53 mutation in both revertants as well as the parental HeLa cells. Sequencing in both orientations of the region corresponding to exons 4-11 of the p53 gene from twelve cDNA clones of each cell line , showed no mutation in

any of the cell lines. p53 mutations in human cancers occur within exons 5-8 which were included in our sequenced regions. Serine 315, phosphorylation of which is necessary for ubiquitin-dependent degradation of p53 (Lin and Desiderio, 1993), was also not mutated in revertants and HeLa.

We have thus hypothesized that the increased p53 protein levels in the HeLa revertants could have resulted in functional p53 activation which would in turn have contributed to or even caused the revertant phenotype. We have therefore tested whether endogenous p53 in the revertants has acquired transcription function properties not manifested in Hela and specific to the wild-type p53 protein. We have used retroviral transduction to establish that p53 function is sufficient to suppress the transformed phenotype of the HeLa cell and we have finally attempted to assess the extent of the contribution of the endogenous p53 to the revertant phenotype.

Exposure to γ-irradiation or Actinomycin D has been shown to induce p53 protein levels and to γ-irradiation has been shown to cause specific induction of the gadd5 and waf-1 genes in cells expressing wild-type p53, but not in cells with mutant p53 (Kastan et al., 1991, 1992 and 1993; El Deiry et al., 1993). We found that in both revertants but not in HeLaEndogenous p53 is inducible by treatment with Actinomycin D and γ-irradiation and active as a constitutive and inducible transcription factor in both revertants but not in HeLa. Exposure to ActinomycinD and γ-irradiation follows different kinetics but induces p53 protein levels to more that ten times that of HeLa at the time point of maximal induction. The kinetic ratesof p53 induction are also more than ten fold higher in the revertants than in HeLa. Notably the HA cell line shows the greatest response to both treatments.

Endogenous transactivational activity in the revertants was able to transactivate the CAT reporter construct pG13 CAT in a p53 dependent manner (Kern et al., 1993). p53 transactivating activity in untreated HF and HA was respectively 30-fold and 20-fold higher than in HeLa. Thus the difference in p53 activity are significantly greater than the difference in the levels of the p53 protein. Transient treatment with Actinomycin D and exposure to γ-irradiation did not significantly increase p53 activity in the HF cell line. Both tretments increased activity in HA by over two-fold and up to 50-fold with respect to HeLa. The observed increase in p53 function after γ-irradiation or ActD (2-fold) was not as dramatic as the observed (10-fold) increase in protein levels but this result is in agreement with a previous report by Zhan et al., (1993). It is possible that due to the multiplicity of the p53 binding sites the pG13 CAT promoter is nearly maximaly transactivated in untreated cells. Alternatively the posttranscriptional modification of the p53 protein that causes its induction may also alter its transactivational effects on the pG13 CAT promoter. Nevertheless these results reinforce other lines of evidence that suggest p53 is better induced in the HA than in the HF revertant.

Gel Shift assays detected p53 binding on a single repeat of the p53 consensus sequence and on the intronic p53 binding sequence of the gadd45 gene upon induction with actinomycin D and γ-irradiation in revertant and positive control but not in HeLa nuclear lysates.

The downstream effectors of the transcriptionally active p53, namely the waf-1 and gadd-45 genes, are expressed in the revertants but not in HeLa. waf-1 mRNA is undetectable in untreated cells and induced upon irradiation of the revertants. waf-1 induction is absolutely dependent upon wild-type p53 function thus this result offers good evidence for the functional activation of the revertant p53. Expression of the gadd-45 mRNA is constitutive in the revertants, and irradiation has a slightly suppressive effect. p53 involvement in gadd45 induction is specific to γ-irradiation and not other treatments (Zhan, 1993 and Kastan 1992), however,
the fold difference in HeLa and revertant gadd45 mRNA is equal to the maximal induction reported for any cell line (RKO cells; Kastan et a., 1992). Thus, gadd45 levels may already be maximally induced in revertants with respect to HeLa gadd45 mRNA levels. p53 specific binding of the gadd45 binding site is detectable in the revertants but not in HeLa and inducible by γ-irradiation and if constitutive gadd45 expression were under p53 control the gadd45 result ould be in agreement with the CAT assay data I is also possible that the γ-irradiation-dependent pathway of gadd45 induction may have been interrupted by mutation of some factor other than p53 in the parental HeLa cell.

Taken together results from CAT assays, DNA binding and endogenous gene expression have established that endogenous revertant p53 protein manifests the transcriptional properteis of the wild-type p53 and is thus shown to be functionally activated with respect to HeLa p53. A strong correlation between the transcriptional activity of p53 and the reversion from cervical carcinoma has thus been established and offers valuable evidence that the transcriptional properties of the wild-type p53 contribute to its role in growth regulation.

In order to establish that wild-type p53 function is sufficient to suppress HeLa transformaation we have used retroviarl transduction to ectopically express wild-type p53 in the revertants. We saw a 100-fold reduction of colony growth in soft agar in the wild-type p53 expressing pull of infected HeLa cells. Similar results have been obtained for tumor cell lines of other origin but not for cervical carcinoma cells. It is actually surprising that the exogenous p53 could have such an effect given the presence of the E6 protein in the Hela cells.

In order to directly assess the extent of the conribution of p53 to the revertant phenotype we have attempted to counteract wild-type p53 function in the revertants by retroviral integration and ectopic expression of a dominant negative mutant p53. Retroviral transduction of the 175 His to Arg mutant did not result to increased clonigenicity of the revertants in soft agar and we are planning to use antisense mRNA to lower the expression of endogenous revertant p53 and definitively prove its contribution to the revertant phenotype. In retrospect, the failure of

dominant negative mutant was expected if endogenous p53 in the revertants had gained wild-type p53 function, since all lines of evidence suggests that in the *in vivo* context wild-type p53 has a dominant effect over the mutants biochemically defined as dominant (see Results section). Thus results from the retroviral transfer and expression of the 175 mutant in the revertants taken together with the manifestation of endogenous p53 function in the revertants are consistent with a dominance of wild-type p53 function *in vivo* in the context of the revertant cells.

We have been interested in identifying the cause of p53 activation in the HeLa revertants. One possible cause for p53 activation would be loss of E6 function. Functional inactivation of E6 in either revertant would ultimately lead to lack/reduction of p53 ubiquitination and degradation. It is also conceivable that interruption of p53 ubiquitination or degradation could occur independent of E6 function and result in p53 activation in the revertants. We have thus used *in vitro* experiments to address whether differential reduction of ubiquitin-dependent degradation in the revertants could account for p53 stabilization. p53 protein was made by *in vitro* translation in reticulocyte lysate extracts. incubated with HeLa or revertant cell lysates in the presence of ubiquitin and ATP or ATP-γ–S. Addition of ATP-γ–S, prevents degradation of high molecular weight ubiquitinated intermediates by the proteasome and allowed us to differentiate ubiquitin dependent degradation from general proteolysis. When we used a partialy purified fraction of basic proteins that contains concentrated HeLa and revertant E6 protein in the degradation reaction mixture we saw that degradation of the *in vitro* translated p53 was faster in the HeLa samples. This result suggested that differential degradation of p53 may be the cause of p53 accumulation in both revertants and the result of loss of E6 function and was in agreement with our retroviral transduction data. We have also observed active inhibition of p53 degradation in the HF cell lysate. Future experiments will dissect in greater detail the ubiquitination-degradation pathways in HeLa and revertants and will aim at identifying the cause of differential p53 degradation in these three cell lines.

Given the evidence suggesting loss of E6 function we have investigated whether E6-p53 interaction was interrupted in the revertants. This was a technically challenging experiment to carry out, given the relatively low abundance of E6 protein. Immunoaffinity purification of p53 complexes from 20mg of HeLa and revertant cell lysates and subsequent immunoblotting with an anti-E6 antibody showed that E6-p53 interaction has been disrupted in the revertant cells. This result definitively identified loss of E6 function as the cause of p53 activation in the revertant cells. Since the loss of E6 function is upstream of E6 p53 binding change within an E6 regulating pathway is the most likely cause of reversion in both HA and HF and we shall actively investigate for differences in the activities of putative E6 regulators.

In an earlier report we have shown that binding of p53 to p34cdc2 occurs in HeLa and HA cells but not in HF cells. p53 is phosphorylated in vivo and in vitro by cdc2p34 (Milner et al.

1990; Sturzbecher et al., 1990; Bischoff et al., 1990) on Serine 315. The predominant NLS is located adjacent to serine 315 (312 for mouse and rat p53). Thus phosphorylation of this serine residue could influence p53 subcellular localization and biological activity in a cell cycle dependent manner. Most importantly, however, Lin and Desiderio have shown that lack of phosphorylation on Serine 315 inhibits the ubiquitin dependent degradation of p53. Thus the observed inhibition of p53 degradation in the HF revertant could be explained by the lack of its interaction with p34cdc2. Since E6 has been reported to bind an unidentified 35 kDa protein it would be interesting to investigate whether E6 also binds p34cdcd2 and whether phosphorylation of p53 on Ser 315 may be required for E6-p53 binding as suggested by our data for the HF cell line. Interestingly, p34cdc2 is also bound by theSV40 Large T Antigen () which like E6 binds and inactivates p53. SV40 binding to E6 leads to increased p53 half-life, and increased p53 phosphorylation (). Cyclin A-cdk2 complexes by SV40 and E1A are able to phosphorylate p107 which is also bound by these oncogenes in the same cell cycle dependent manner than they would if they were not bound by the viral oncogenes. It is thus possible that when bound to SV40 Large T p53 may be phosphorylated by p34cdc2 and this interaction may be important for p53 inactivation by SV40 Large T. The lack of p34cdc2 -p53 interaction in the HF cell line may thus explain why SV40 Large T fails to retransform HF. In contrast, SV40 Large T can retransform HA and p34cdc2 -p53 interaction is preserved in the HA revertant while E6-dependent degradation of p53 is blocked. Thus p34 cdc2 may be a common regulator of two biochemically distinct pathways through which SV40 Large T and E6, each exert their inactivating effects on p53. In the HF revertant inactivation of p34 cdc2 may disrupt the function of both viral oncogenes while in the HA revertant SV40 Large T can still exert its inactivating effect on p53. Future experiments will attempt to verify these hypotheses.

In summary, completed work establishes that p53 which can suppress the tumorigenicity of the HeLa cell, is functionally activated in the revertants. Loss of E6 function and of ubiquitin dependent degradation have been identified as the cause for functional p53 activation and a testable hypothesis has been formulated about the involvement of p34cdc2 in the functional pathway of the E6 viral oncogene. These results point to the value of this system for the study of cervical cell transformation and show great promise for advancing our understanding of p53 and viral oncogene function.

SIGNIFICANCE AND FUTURE EXPERIMENTS

This Research Thesis describes the initial characterization of non-transformed revertants isolated from HeLa cells on the basis of prolonged rhodamine 123. Initial phenotypic characterization of the revertants demonstrated that these cells lost most of the *in vitro* properties associated with transformed cells, including a transformed morphology, clonigenicity in semi-solid medium, and growth in suspension culture (Boylan et al, 1996). Stanbridge and co-workers have previously carried out somatic cell hybridization experiments using HeLa cells and primary human fibroblasts and showed that the tumorigenicity of HeLa cells is suppressible (). They have futher demonstrated that a tumor suppressor gene harbored on the short arm of human chromosome 11, is able to suppress the tumorigenicity of HeLa cells (Maniatis et al., 1982; Srivatsan et al, 1986 ; Stanbridge et al., 1986). However, all of the non-tumorigenic cell hybrids generated from the fusion of HeLa cells and normal human fibroblasts invariably maintained the *in vitro* characteristics of the transformed parental Hela. Thus, loss of the tumor suppressor gene on chromosome 11 is likely to be associated with tumor progression rather than initiation of carcinogenesis. In contrast, the events that led to reversion in our system may have affected early pathways of transformation, such as E6 and E7 functional pathways. In rodent transformed cell / revertant cell systems reversion is often the cause of inactivatng mutation within the transforming oncogene (). Our knowledge of the involvement of E6 and E7 in cervical transformation makes each of these genes the analogue of the transforming oncogene in the rodent systems. It was therefore necessary to examine the possibility of functional inactivation of each of the two oncogenes.

Continued expression of the E6/E7 genes -which has been found necessary for the maintenance of the transformed phenotype of the HeLa cell (Schwarz et al., 1985; Schneider-Gadicke et al., 1986; Schneider-Gadicke et al., 1987; Bosch et al., 1990; Bosch et al., 1991)- was verified in the revertants. Retroviral transduction of HPV-16 E6, E7 or a combination of the two had no effect on the revertant phenotype. On the other hand, cell fusion experiments between HeLa and revertants raised the possibility that a tumor suppressor gene may have been activated during the reversion process. Since the transforming functions of the "high risk" HPV E6 and E7 are thought to result from their respective binding and inactivation of the p53 and Rb tumor suppressor proteins (Dyson et al., 1989; Munger et al., 1989; Munger et al., 1989; Werness et al., 1990) functional inactivation of the E6 and/or E7 could be manifested as activation of either Rb or p53 tumor suppressors. Such activation could be caused by a change in a regulator of either tumor suppressor or of the relevant HPV oncogene protein and a subsequent interruption of the association between the viral oncoprotein and cellular tumor suppressor. We have thus examined the state of the p53 and Rb tumor suppressor genes in HeLa and revertant cells.

We found increased steady state levels of wild-type p53 in both revertants despite the presence of the E6 protein. Detectable Rb-E2FI binding in the revertants suggested functional Rb

activation. In addition, qualitative changes in protein-protein interactions in both the Rb and p53 pathways were observed in the HF revertant. To this point our data had established that reversion of transformation was the result of functional inactivation of the E6 and E7 pathways in the revertants. Increase of the levels of p53 was the most consistent and striking difference between HeLa and the revertants and the remainder of the research presented in this report focused on the study of the p53 gene product in our system. We have thus shown that wild-type p53 can suppress the HeLa phenotype. We have further succeeded in establishing that revertant p53 is wild-type and that wild-type-p53-specific functions are manifested in the revertants but not in HeLa. Finally, we have shown that decreased p53 proteolysis is the probable cause of increased p53 levels in the revertants with respect to HeLa. Loss of E6 function and of ubiquitin dependent degradation have been identified as the cause for functional p53 activation and a testable hypothesis has been formulated about the involvement of p34cdc2 in the functional pathway of the E6 viral oncogene. Although the extent of p53 contribution to the revertant phenotype remains to be assessed, the research presented here emphasizes the central role of the HPV and p53 pathways in cervical transformation and offers *in vivo* evidence for the link between the transcriptional activity of p53 and the control of cell growth. These results point to the value of this system for the study of cervical cell transformation and illustrate its great promise for advancing our understanding of p53 and viral oncogene function. Most importantly, this work suggests that interference with the HPV functional pathway or activation of the p53 pathway may help control cervical carcinoma cell growth and may be a reasonable approach to the development of new therapeutic strategies. The use of transformant/revertant human carcinoma cell systems isolated for loss of prolonged rhodamine retention is thus validated as a more general approach to the study of human carcinoma cell transformation.

The failure of dominant negative p53 mutant to retransform the revertants is consistent with activation of p53 in these cells, since all lines of evidence suggests that in the *in vivo* context wild-type p53 has a dominant effect over the dominant negative mutants. Although other lines of experimentation strongly corroborate this interpretation, it would be desirable to test the extent of p53 contribution to the revertant phenotype in each revertant. We are thus planning to use antisense mRNA to lower the expression of endogenous revertant p53 and definitively prove its contribution to the revertant phenotype.

p53 activation is attributed to loss of E6 function upstream of E6-p53 binding. Change within an E6 regulating pathway is the most likely cause of reversion in both HA and HF. We shall thus investigate differences in the activities of putative E6 regulators.

Decreased binding of p53 to p34cdc2 in HF cells may have been caused by mutation in p34cdc2 itself or by its inactivation by a molecule such as the cyclin activating kinase (CAK; for

review see Solomon, 1995). Either inactivation of p34cdc2 or functional activation of CAK would have profound effects in the cell cycle. It thus becomes imperative to study the significance of the interaction of p34cdc2 and p53 as well as the cause for its absence in the HF cell. We shall therefore check whether changes in p34cdc2 activity are manifested in the revertants by immunoprecipitation of p34cdc2 followed by kinase activity assay in immunoprecipitated complexes. We shall use functional assays such as those performed for viral oncogenes and p53 to test whether mutation of p34cdc2 or differential CAK activity can account for differences in p34cdc2 function. Furthermore, in order to investigate the significance of p34cdc2-p53 interaction we shall examine its cell cycle and DNA damage dependence and the codependence of p34cdc2 activity and p53 phosphorylation. The performance of these experiments in HeLa revertants and control primary human keratinocytes and Saos-2 cells will allow us to establish a cause-and-effect relationship in the p53-p34cdc2 association.

Lin and Desiderio have shown that lack of phosphorylation on Serine 315, the p34cdc2 substrate, inhibits the ubiquitin dependent degradation of p53. Thus the observed inhibition of p53 degradation in the HF revertant could be explained by the lack of its interaction with p34cdc2. Since E6 has been reported to bind an unidentified 35 kDa protein it would be interesting to investigate whether E6 also binds p34cdcd2 and whether phosphorylation of p53 on Ser 315 may be required for E6-p53 binding as suggested by our data for the HF cell line. Interestingly, p34cdc2 is also bound by theSV40 Large T Antigen () which like E6 binds and inactivates p53. SV40 binding to E6 leads to increased p53 half-life, and increased p53 phosphorylation. Cyclin A-cdk2 complexes by SV40 and E1A are able to phosphorylate p107 which is also bound by these oncogenes in the same cell cycle dependent manner than they would if they were not bound by the viral oncogenes. It is thus possible that when bound to SV40 Large T p53 may be phosphorylated by p34cdc2 and this interaction may be important for p53 inactivation by SV40 Large T. The lack of p34cdc2 -p53 interaction in the HF cell line may thus explain why SV40 Large T fails to retransform HF. In contrast, SV40 Large T can retransform HA and p34cdc2 -p53 interaction is preserved in the HA revertant while E6-dependent degradation of p53 is blocked. Thus p34 cdc2 may be a common regulator of two biochemically distinct pathways through which SV40 Large T and E6, each exert their inactivating effects on p53. In the HF revertant inactivation of p34 cdc2 may disrupt the function of both viral oncogenes while in the HA revertant SV40 Large T an still exert its inactivating effect on p53. Future experiments will attempt to verify these hypotheses.

Finally, we shall investigate the role of p53 in cell death or growth arrest upon induction with Actinomycin D and γ-irradiation in the context of the revertant cells. We shall compare revertants with Hela cells expressing wild-type p53 in order to assess the contribution of p53 to these response in the revertants. These cell cycle studies will provide further evidence for the

contribution of the p53 in the revertant phenotype, but they may also point out differences in the functional pathways involved in reversion and p53 activation of each cell line.

Metabolic labeling and immunoprecipitation of Rb protein from HeLa and revertants suggested that their may be differences in the Rb phosphorylation potential of revertants and are currently investigating this hypothesis. Rb changes in phosphorylation may account for the co-immunoprecipitation of Rb with E2FI from revertant cell lystates that suggests Rb activation.

Although Rb-E2FI complex formation suggests Rb activation in the revertants a more definitive proof of gain of Rb function would be given by a direct indication of a suppressive effect of Rb on E2FI function as measured in transcription and DNA binding assays in the revertants. The extent/dose of functional Rb activation may be important to Rb contribuion in the reversion process as may be the interplay between Rb and p53 gain of function in the revertants. We are currently investigating whether complex formation between Rb and E2F-1 could account for differential E2F-1 activity in the revertants and what changes in Rb or E2F regulation can account for the restoration of complex formation in HF. We are also testing the hypothesis that gain of function of Rb through this complex may have contributed to the reversion (Hiebert et al., 1993). E2F1-Rb association is transient in the cell cycle and dependent on Rb phosphorylation by cell cycle regulatory complexes (Schwarz et al., 1993 ; Shirodkar et al., 1992; Hiebert et al, 1993; Nevinset al., 1992) An investigation of the Rb phosphorylation mechanisms in the revertants may identify the reversion event among the most basic mechanisms of cell cycle and growth control.

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