DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

1

STUDIES ON HUMAN ONCOPROTEIN p53

by

TOIVO MAIMETS

TARTU 1991

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MAIN REFERENCES

This thesis is summary of the following communications which will be referred to by Roman numerals given below:

I. Майметс Т.О. и Дженкинс Дж.Р. (1987) Модифицированный онкобелок p53 в опухолевых клетках линии НТ 1080 человека. Доклады Академии Наук СССР 296, 757-759.

II. Майметс Т.О. (1988) Бактериальная экспрессия человеческого онкогена p53 в форме слитого белка. Доклады Академии Наук СССР 302, 1501-1503.

III. **Maimets T., Stürzbecher H.-W., Brain R., Court W. and Jenkins J.R. (1990) The interaction of SV40 transcription enhancer with human oncoprotein p53. Manuscript.**

IV. **Stürzbecher H.-W., Brain R., Maimets T., Addison C., Rudge K. and Jenkins J.R. (1988) Mouse p53 blocks SV40 DNA replication in vitro and downregulates T antigen DNA helicase activity. Oncogene 3, 405-413.**

V. **Jenkins J.R., Stürzbecher H.-W., Brain R., Grimaldi M., Maimets T., Rudge K., Court W. and Addison C. (1989) Analysis of human p53 mutants that are trans-dominant modulators of DNA replication in vivo. Cancer Cells 7, 127-135.**

VI. **Stürzbecher H.-W., Maimets T., Chumakov P., Brain R., Addison C., Simanis V., Rudge K., Philp R., Grimaldi M., Court W. and Jenkins J.R. (1990) p53 interacts with $p34^{cdc2}$ in mammalian cells: implications for cell cycle control and oncogenesis. Oncogene 5, 795-801.**

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KOKKUVõTE

Käesolevas väitekirjas kirjeldatakse eksperimentaalseid uurimistöö tulemusi, mis autor on saanud inimese onkovalgu p53 ekspressiooni ja tema rakusiseste molekulaarsete toimemehhanismide kohta.

Onkovalk p53 on onkogeeni p53 valkprodukt ja tihedalt seotud rakkude transformatsiooni ja tumorigeneesiga. Ta avastati viirusega SV40 transformeeritud rakkudest ja hiljem on näidatud tema seos mitmete teiste transformeerivate viirustega (adenoviirus, papilloomiviirus). Mitmete kasvajavormide puhul täheldatakse p53 suurenenud produktsiooni rakkudes, samuti esineb kasvajadiagnoosiga patsientidel antikehi selle valgu vastu.

Algselt klassifitseeriti p53 kui nukleaarset onkogeeni, kuna ta võib immortaliseerida primaarseid rakke ja koopereeruda aktiveeritud ras-onkogeeniga rakkude transformatsioonil. Samas on ka veenvaid tõendeid selle kohta, et p53 võib käituda nn. "anti-onkogeenina" e. "retsessiivse onkogeenina". Selline mitmesuunaline aktiivsus osutab võimalusele, et p53 enda tegevust moduleeritakse rakus mitmeti.

Üsna vähe on teada valgu p53 funktsioonist normaalseis rakkudes. Mitmed andmed osutavad selle lühikese elueaga fosfovalgu tähtsusele DNA replikatsioonis, ent on ka andmeid p53 võimaliku funktsiooni kohta RNA transkriptsiooniprotsessis.

Uurimaks onkovalgu p53 funktsiooni normaalses raku proliferatsioonis ja tumorigeneesis püstitasime me järgmised küsimused:

-Kuidas ekspresseeritakse vaiku p53 erinevais rakkudes?

-Milline on p53 toimekoht rakutsüklis?

-Mis on p53 täpseks molekulaarseks toimemehhanismiks rakkude normaalse kasvu ajal?

-Mismoodi häiritakse seda mehhanismi tumorigeneesi puhul?

-Kuidas reguleeritakse p53 tegevust rakutsükli vältel?

Esitatav töö koosneb kahest sisuliselt väga seotud osast. Kuna kogu maailmas puudusid vahendid inimese p53 molekulaarbioloogiliseks uurimiseks, tuli alustada nende väljatöötamisest. Esimene osa tööst kirjeldabki nn. tehniliste vahendite (inimese valgu p53 tootmine bakterirakkudes, antiseerumite valmistamine, inimese p53 produtseerimine putukarakkudes) loomist. Terve kompleksi uute molekulaarbioloogiliste meetodite rakendamine võimaldas luua uurimisvahendite baasi, mis oli hädavajalik p53 funktsiooni uurimiseks ja andis meile selles mõttes olulise tehnilise eelise teiste laborite ees. Esmakordselt õnnestus saada inimese p53-spetsiifilisi antikehasid ja

toota seda looduslikult väheesinevat valku biotehnoloogiameetoditega suurtes kogustes.

Töö teine osa kirjeldab p53 funktsionaalseid uuringuid - s.t. seda, milleks me eelnevalt saadud uudseid vahendeid kasutasime ja milliseid tulemusi saime.

Et leida erinevusi p53 ekspressioonis erinevatel transformatsiooniastmetel olevais rakuliinides, uurisime me mitme meetodiga inimese rakuliine HT1080, RPMI5966, HeLa jt. ja avastasime immunoloogilisi kõrvalekaldeid p53 ekspressioonis. Me püstitasime hüpoteesi, et sellised kõrvalekalded võivad peegeldada p53 rakusiseseid interaktsioone, mis vahendavad p53 funktsiooni ja võivad olla p53 regulaatoreiks (see olekski nn onkogeenide aktiveerumise või inaktiveerumise mehhanism). Üheks edaspidiseks töösuunaks oligi selliste interaktsioonide tuvastamine.

Leidmaks vastust küsimusele, millises rakutsükli etapis võiks p53 funktsioneerida, kasutasime me in vitro SV40 DNA replikatsioonisüsteemi ja transkriptsioonisüsteemi. Ehkki need ei peegelda täiesti täpselt vastavaid protsesse rakulise DNA-ga, on need siiski enamkasutatavad mudelid vastavaiks uuringuiks, kuna ainsa viirusvalguna osaleb sel puhul SV40 suur T antigeen ja ülejäänud komponendid on rakulised. Me näitasime, et mõned p53 vormid inhibeerivad efektiivselt SV40 DNA replikatsiooniprotsessi selle alguses, s.t. initsiatsioonifaasis. Samal ajal ei olnud p53-l mõju ei SV40 varajasele ega hilisele transkriptsioonile.

Et T antigeen on ainus vajalik viirusvalk SV40 replikatsiooniks ja on teada, et ta seostub valguga p53, uurisime me kõigepealt p53 mõju T antigeeni omadustele. Osutus, et p53 küll veidi takistas T antigeeni seondumist replikatsiooni aiguskohta (ori) ja vähendas teatud määral T antigeeni helikaasset aktiivsust, ent need fenomenid ei olnud piisavad, seletamaks täielikult p53 efekti replikatsioonis. Veel enam, meil õnnestus konstrueerida p53 mutantseid vorme, mis küll suure T antigeeniga interageerusid, ent DNA replikatsiooni ei mõjutanud. Seetõttu me postuleerisime, et eksisteerib veel mingi p53 aktiivsus, mis on sõltumatu SV40 T antigeenist ja mis annab panuse p53 funktsioonile SV40 DNA replikatsioonis.

Edasised uurimused näitasid, et p53 on tõepoolest võimeline ka otse interageeruma SV40 üheahelalise DNA-ga replikatsiooni-transkriptsiooni regulaatorpiirkonnas. õnnestus ka välja selgitada see piirkond SV40 DNA-l, mis selles interaktsioonis osaleb. Me järeldame, et see DNA järjestus on üks molekulaarne märklaud (lisaks T antigeenile), mille kaudu p53 moduleerib SV40 replikatsiooni ja teeb seda ilmselt üheahelalise DNA struktuuri stabiliseerimise kaudu pärast DNA ahelate lahtikeeramist T antigeeni helikaasse

aktiivsuse poolt. Me arvame, et see peab paika ka rakulise DNA replikatsiooni puhul, ehkki vastavaist helikaasidest on väga vähe teada.

Paljudes eri tüüpi kasvajates on leitud, et p53 geen sisaldab kas ühes või mõlemas alleelis punktmutatsioone ja on arvatud, et see võib olla üheks kasvajate tekke molekulaarseks põhjuseks. Me uurisime, kas sellised punktmutatsioonid kuigivõrd peegelduvad ka p53 muutunud aktiivsusena DNA replikatsioonis. Osutus, et isegi väga väikesed muutused p53 geeni struktuuris, mis tingivad vaid ühe aminohappe muutuse 393-st, võivad viia väga suurtele p53 aktiivsuse erinevustele DNA replikatsioonis. Need tööd viivad kahele järeldusele: esmalt osutavad nad tõepoolest p53 otsesele rollile DNA replikatsiooni ensümoloogias. Teisalt on selge, et sellised muutused võivad tõesti olla p53 erineva funktsiooni aluseks ja seega põhjustada p53 olekut "aktiveeritud onkogeenina" või "anti-onkogeenina".

Üks meie uurimissuund oli püstitatud eesmärgiga leida viiruseliste p53-seoseliste valkude rakulisi analooge, mis võiksid olla kas p53 aktiivsuse märklauaks või seda reguleerida. Meil õnnestus näidata, et üks universaalne raku regulaatorvalk, $p34^{cdc2}$, on võimeline nii in vivo kui in vitro seonduma inimese onkovalguga p53. $p34^{cdc2}$ omab proteiinkinaasset aktiivsust ja me näitasime, et ta suudab spetsiifiliselt fosforüleerida p53 aminohappejääki Ser-315. Selline aktiivsus on enim väljendunud rakkude S-faasis. Neist andmeist tulenevalt pakkusime me välja hüpoteesi, mille kohaselt p53 aktiivsus replikatsioonis on rakutsükli kestel kontrollitav valgu $p34^{cdc2}$ poolt. Selle kohaselt on G_1-faasis olevates rakkudes p53 Ser-315 fosforüleerimata ja seetõttu p53 replikatsioonis inaktiivne (või aktiivne suppressor). p53 fosforüleerimine S-faasis $p34^{cdc2}$ poolt võimaldab DNA replikatsiooni toimumise.

Käesoleva väitekirja põhiseisukohad on kirjeldatud viies avaldatud teadusartiklis ja ühes avaldamisel olevas käsikirjas.

A. INTRODUCTION

The early 1980-ies, when we in Tartu, initiated by late Prof. Artur Lind (1927-1989), started studies on oncogenes, were extremely interesting years to join this field. Most of the known oncogenes had been already discovered and investigators started to move from descriptive phase into asking questions about molecular mechanisms of functioning of particular oncogenes and their role in molecular events underlying normal cell proliferation and tumorigenesis.

Because of several lucky coincidences and also the fact that nuclear oncogenes seemed to be much more handy objects to ask really questions on the molecular level of action, I started to be interested in cellular oncogene p53 and, more specifically, in the role of its protein product in molecular events of the cell cycle. At that time very little was known about human p53 protein and I tried to concentrate (with the help of model experiments in other systems) to this species.

The main questions we asked from the very beginning were:

- How is p53 expressed in different cells?
- What is the point of action of protein p53 in cell cycle?
- What is the precise mechanism of p53 action in normal cellular growth?
- How can this mechanism be disturbed by mutations very often found in tumor cells?
- How is the functioning of p53 regulated throughout cell cycle?

My present thesis is a description of how many answers to these questions we have today.

This thesis consists, in fact, of two very much related parts. First I describe how we generated principally new reagents to study human p53 (bacterial protein, antisera, purified eukaryotic protein). Then I show how we used these reagents for functional studies of human p53 and what we found.

ONCOGENE p53 AND ITS EXPRESSION

The cellular p53 protein was first detected in SV40 transformed cells by its ability to form a stable complex with the SV40 large T antigen (De Leo et al.,1979, Kress et al., 1979, Lane and Crawford 1979, Linzer and Levine,1979, Rotter et al.,1980). Similarily in cells transformed by adenovirus type 5, it was shown that the viral E1B 58 kD protein binds and stabilizes protein p53 (Sarnow et al.,1982a,b).Later it was found that many transformed cell lines and human tumor cells from patients with various forms of tumors contain an elevated level of p53, whereas nontransformed cells contain only small amount of the protein and that antibodies produced by certain tumor-bearing animals were able to immunoprecipitate p53 from several mouse neoplastic cell lines (Kress et al.,1979, Melero et al.,1979, Rotter et al.,1980). Anti-p53 activity was also detected in sera from cancer patients (Crawford et al.,1982, de Fromentel et al.,1987).

This protein was originally classified as a nuclear oncogene since gene transfer experiments indicated that p53 was able to both extend the lifespan of primary rodent cell cultures and to cooperate with the activated H-ras oncogene in transforming these cells (Eliyahu et al.,1984, Jenkins et al.,1984, Parada et al.,1984). Some mutant p53 proteins have greatly enhanced activity in such transformation assays indicating that p53 can be activated by mutations (Jenkins et al.,1985, Finlay et al.,1988). At the same time there is conclusive evidence that the loss of normal p53 expression and function may constitute an important step in the transformation process (Mowat et al.,1985, Chow et al.,1987, Rovinski et al.,1987, Ben-David et al.,1988, Munroe et al.,1988), suggesting that p53 may act as a "recessive oncogene" or "tumor suppressor gene". In either event the activities of p53 as oncogene or anti-oncogene product are likely to impact upon the cell via interactions with other cellular proteins.

So far the gene for p53 has been found from mouse, human, monkey, rat, chicken, Xenopus laevis and rainbow trout cells (for detailed review see Jenkins and Stürzbecher,1988 and Soussi et al.,1990). Both mouse and human genomes contain a single copy of a functional p53 gene per haploid genome where it is located on chromosome 11 (Czosnek et al.,1984, Rotter et al.,1984) and the short arm of chromosome 17 (Benchimol et al.,1985, Isobe et al.,1986, McBride et al.,1986, Miller et al.,1986), respectively. Both genes contain 11 exons interrupted by 10 introns, whereas exon 1 preceding to a very large intron 1 (10 kB in human and 6.1 kB in mouse cells) is a non-coding sequence.

Northern blot analysis revealed that p53 mRNA from different species is 2-3 kB long (2.0 for mouse and 2.8 for human cells). It has been shown that the level of p53 mRNA is elevated in some tumors (Rogel et al.,1985), undifferentiated stem cells, embryonal carcinoma cell line F9, murine erythroleukaemia cells (Reich et al.,1983, Bendori et al.,1987) as well as during mouse organogenesis (day 9 to 11) (Rogel et al.,1985) and chicken embryonic development (Louis et al 1988).

p53 PROTEINS

In untransformed cells p53 protein is extremely unstable with a half-life of only 6 minutes in spleen tissue (Rogel et al.,1985) and 20 to 30 minutes in most cell lines (see Crawford, 1983, Oren,1985, Rotter and Wolf,1985, for reviews). In transformed cells p53 is generally more stable than in untransformed cells with a half-life of about 5 hours in chemically transformed cells(Reich et al., 1983) and more than 20 hours in SV40-transformed cells (Oren et al., 1981). It has been suggested that complex formation with T-antigen increases the stability of p53 in SV40-transformed cells (Oren et al.,1981). Later studies have shown, however, that p53 stabilization in SV40-transformed cells rather occurs due to indirect changes in the mechanisms controlling the half-life of p53 (Deppert and Haug,1986, Deppert et al.,1987). In some cases increased stability of p53 appears to be a consequence of changes in protein structure. Jenkins et al(1985) have found that in rat cell lines immortalized by constructs encoding the p53 mutant dl162 the endogenous rat p53 had a half-life of 4 hours while the dl162 mutant protein had half-life of 24 hours.

p53 is located predominantly in the cell nucleus (Dippold et al.,1981, Gurney et al.,1980, Rotter et al.,1983) and nuclear location signals have been defined in the amino acid sequence of this protein (Addison et al.,1990, Shaulsky et al.,1990). Recent findings indicate that active protein synthesis is needed in cells to locate p53 in cell nucleus (Gannon and Lane,1991).

Comparison of all known p53 sequences (Soussi et al.,1990, see fig.1) revealed that five clusters of amino acids (domains I to V) are highly conserved through evolution. The sequences linking these domains are much more divergent, reflecting that these regions are probably not involved in essential functions. The only posttranslational modification known for p53 so far is phosphorylation on multiple serine and threonine residues (Van Roy et al.,1981, Meek and Eckhart,1988). Furthermore, a small RNA with unknown

function was found to be covalently linked to serine 386 at the C-terminus of murine p53 (Samad et al.,1986).

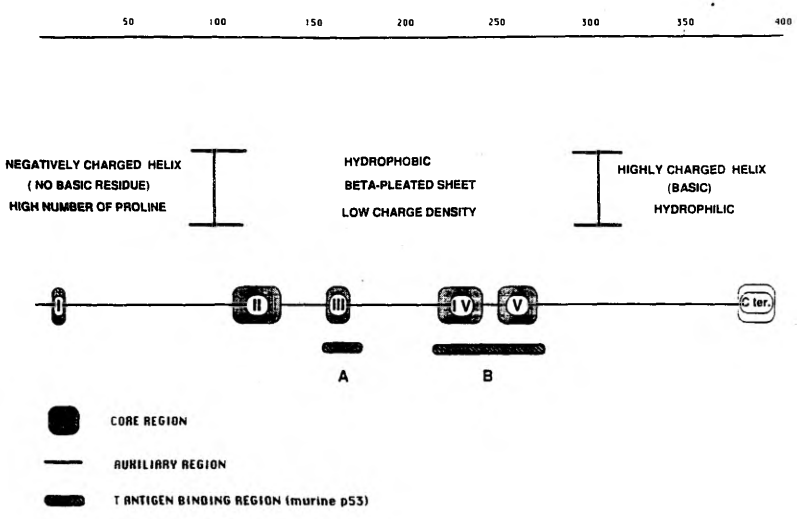

Figure 1 Scematic representation of p53. Top:p53 chain conformation . Bottom: A scematic p53 molecule showing the relative positions of the hihgly conserved domains (I to V) and the p53 binding sites (A and B) to the SV40 large T antigen (by Soussi et al.,1990).

VIRAL AND CELLULAR TARGETS OF p53

The most prominent biochemical property of p53 is its ability to interact with a variety of proteins of viral and cellular origin. In addition to that, p53 is able to assemble into homologous oligomeric structures (Kraiss et al.,1988, Burger and Fanning,1983).

The best-characterized biochemical feature of p53 is the formation of a heterologous complex with SV40 large T antigen. Two discontinous regions of p53 were shown to be implicated in it (Jenkins et al.,1988) and this property of p53 is highly conserved in evolution (see Soussi et al.,1990), indicating that this association could play role in functioning of p53.

Sarnow and co-workers have identified heterologous complexes between p53 and the E1B 58 kD protein from Ad2 and Ad5 (Sarnow et al., 1982a, Sarnow et al.,1982b), whereas such complexes are found in adenovirus transformed but not infected cells (Sarnow et al.,1984).

A number of laboratories have reported the formation of complexes between mouse and primate p53 and members of the 70 kD family of heat shock proteins (Pinhasi-Kimhi et al.,1986, Hinds et al.,1987, Stürzbecher et al.,1987, Erhart et al.,1988). This property seems to be restricted to mutated forms of p53 and interestingly these mutant forms bind either poorly or not at all SV40 large T antigen.

Recently one more viral transforming protein has been shown to bind p53: E6 protein of human papilloma viruses HPV16 and HPV18 (Werness et al.,1990). This association stimulates the degradation of p53 in cells (Scheffner et al.,1990).

p53 has been also shown to bind double-stranded as well as single-stranded DNA (Lane and Gannon,1983, Steinmeyer and Deppert,1988, Kern et al.,1991) without showing any sequence specificity requirements on DNA.

PROPOSED FUNCTION(S) OF p53

A number of lines of evidence link p53 to cell-cycle related events. Reich and Levine(1984) showed that expression of p53 was cell-cycle regulated with maximal expression in late G_1. A role for p53 as an essential factor for continous cell proliferation has been suggested by studies including antibody microinjection (Mercer et al.,1982) and transfection with plasmids encoding p53 anti-sense RNA (Shohat et al.,1987, Prasolov and Chumakov,1989). Recent data from Deppert's group strongly argue for p53 being a protein controlling transition of cells through R-point from G_1 to S phase, acting as a "gatekeeper" in the R-point and exerting a positive effect on the transition of cells through the cell cycle (Steinmeyer et al.,1990, Deppert et al.,1990).

Suggestive evidence for a role of p53 protein in DNA replication was provided by the findings that mouse p53 is able to compete both with DNA polymerase alpha for binding to the SV40 T antigen (Gannon and Lane,1987) and to inhibit SV40 origin-dependent DNA replication in monkey COS cells (Braithwaite et al.,1987). In addition to that, p53 is found in "replication compartments" of herpes-infected cells (Wilcock and Lane,1991) indicating the role of p53 in HSV-1 DNA replication as well as in DNA replication of normal,

uninfected cell. However, p53 is clearly not an absolute requirement for DNA synthesis since at least two viable cell lines L12 (Wolf et al.,1984) and HL-60 (Wolf and Rotter,1985), are reported to lack p53.

Another line of (although somewhat indirect) evidence argues for the role of p53 in RNA transcription process. Hybrid proteins that contain the DNA binding domain of yeast GAL4 and portions of p53 have been used to show that the p53 protein contains a transcription-activating sequence (Fields and Jang,1990, Raycroft et al.,1990, O'Rourke et al.,1990) and this property is inherent to 73 N-terminal amino acids of p53.

The importance of central domains II to V in p53 function is strongly supported by different laboratories: i) A number of mutations of murine p53, located in this region, result in the activation of p53 for cooperation with the activated H-ras oncogene (Finlay et al.,1988, Eliyahu et al.,1988, Hinds et al.,1989) ii) The p53 gene is frequently rearranged in malignant cell lines derived from the spleen of Friend virus infected mice. A number of these rearrangements involve the highly conserved domains II to V of p53 (Mowat et al.,1985, Chow et al.,1987, Ben-David et al.,1988, Munroe et al.,1988). In each case, rearrangement of these regions leads to a mutated form of the protein p53 which acts as a oncogene. iii) The majority of the point mutations identified in diverse human tumor types (Baker et al.,1989, Nigro et al.,1989, Takahashi et al.,1989, Malkin et al.,1990, Srivastava et al.,1990, Cheng and Haas,1990, Bressac et al.,1990, Mulligan et al.,1990, Rodrigues et al.,1990), although not all of them, also involve the conserved domains, reinforcing the idea that these domains may be the preferential target of transformation-associated rearrangements.

B. THE PRESENT INVESTIGATION

EXPRESSION OF EUCARYOTIC p53 IN BACTERIAL CELLS (II)

To express human p53 in bacterial cells we choose expression vector pATH (II, fig.1), which contains part of anthranylate synthetase gene (trpE) coding 336 N-terminal amino acids of this enzyme under the control of tryptophane promotor (P). The latter is suppressed by tryptophane and is inducible by indolepropionylic or indoleacrylic acids. Polylinker site (PL) after trpE makes it possible to clone desired coding sequences into this vector. If the sequence is in frame with trpE, the bacterial expression product from such a construct is a fusion protein containing 336 amino acids from trpE and the C-terminal part corresponding to the cloned sequence.

Our first approach was to clone 842 bp BalI-SmaI fragment of human p53 cDNA (Harlow et al.,1985) corresponding to C-terminal amino acids 161-393 into PL site of vector pATH11. Expression of such recombinant vector in E. coil cells gave good production of fusion protein trpE-hp53 (II fig.2) which was recognizable by p53-specific monoclonal antibodies pAb421. As the epitope for pAb421 in p53 lies in the very C-terminus of p53 (Wade-Evans and Jenkins,1985), we conclude that the fusion protein contains the entire cloned p53 sequence. The resulting fusion protein could be purified by gel filtration and such protein bound both p53-specific and trpE-specific monoclonal antibodies (II fig.3).

In another set of experiments we expressed human and Xenopus p53 in bacterial cells cloning the entire coding sequence (Harlow et al.,1985 and Soussi et al.,1987, respectively) into PL site of pATH with the help of polymerase chain reaction (PCR) technique (Scharf et al.,1986). The corresponding fusion proteins were purified by SDS-denaturing gel electrophoresis and injected into rabbits. As a result we got highly species-specific antisera against human p53 and Xenopus p53. These antisera made it possible to study the expression and mode of action of p53 in corresponding cells.

Functional studies of bacterially produced p53 proteins showed, however, that although these proteins are good antigens for making specific antibodies, they do not reflect many other properties, inherent to native eukaryotic p53 (oligomerization, binding to several viral proteins etc.). Therefore they could not be used as model substrates to study mechanisms of p53 functioning. For this type of studies we undertook production of human p53 in eukaryotic cells (see below).

STUDIES ON THE EXPRESSION OF p53 IN DIFFERENT HUMAN CELL LINES (I)

Because the cellular protein p53 has been for many times shown to be linked to cellular transformation (see introduction and references therein), one of our first approaches to study p53 in human cells was to investigate expression of this protein in established human cell lines which are at different levels of transformation (RPMI 5966, HT 1080, HeLa). The idea was that if cellular transformation is caused by abnormalities (quantitative or qualitative) in p53 expression, one could detect the differences either at the level of transcription, translation or posttranslational events. The methods we used in these studies (Southern and Northern blottings, immunoprecipitations, half-life measurements) were not sensitive enough to detect the changes at the level of single point mutations (these methods became available later). They were, however, good enough to see bigger changes in p53 gene and its expression. As a result of these studies, we could show (I) that in human fibrosarcoma cell line HT 1080 there is abnormal expression of p53. Although the gene structure as well as amount and quality of corresponding mRNA is normal (I fig.2), the protein from HT 1080 could not be immunoprecipitated by pAb421 recognizing the C-terminus of p53 (I fig.1). Precipitations with polyclonal antisera showed, however, that the protein was normally translated (I fig. 1) and the corresponding epitope is masked by interactions with other cellular components. The real presence of pAb421 epitope in HT 1080 cells was first confirmed by Southern and Northern blotting experiments and later by direct sequencing of p53 genomic DNA (Chumakov et al., unpublished). Furthermore, we could show that such an interaction of p53 in HT 1080 cells leads to considerable increase of its half-life (about 4 hours, I fig. 3). Such an increase in p53 half-life due to interactions with other proteins was already known for p53 - SV40 T complexes (Oren et al.,1981).

In much less transformed human melanoma cell line RPMI 5966 we did not see any gross abnormalities (within the limits of the sensitivity of methods).

The expression of p53 in human HeLa cells had been an intriguing question for some time. We found that although there is a normal gene for p53 present and the level of transcription is even somewhat higher than in other cells, no protein could be immunoprecipitated neither by monoclonal antibodies nor by antisera. Using our human p53-specific antisera we could only recently demonstrate (Strzbecher et al., International p53 Workshop,

1990) that there are indeed very minute amounts of p53 protein in these cells. It has been shown now that p53 is rapidly degraded in these cells due to interaction with HPV E6 protein (Scheffner et al.,1990).

The studies of p53 expression in different transformed human cell lines led us to the idea that there can be other cellular proteins which, by interacting with p53, can regulate its functional activity. Such a modulation of oncoprotein activity could be , in fact, the mechanism of oncogene activation. Therefore, one of our next projects was to detect cellular proteins which interact with p53 and can therefore be its regulators (see below).

EXPRESSION OF HUMAN p53 IN INSECT CELLS USING BACULOVIRUS EXPRESSION VECTORS (III, IV)

To produce functionally active human p53 protein we expressed it in insect Spodoptera frugiperda cells using recombinant nuclear polyhedrosis virus containing p53 cDNA under polyhedrin promotor (Summers and Smith,1987). Human oncoprotein p53 was immunopurified from these cells on antibody pAb421-protein A Sepharose column. This way of protein purification was mild and did not contain any denaturing steps (extreme pH, use of detergents etc.).

Our first question was whether such a protein is biochemically identical to the protein produced by primate cells. As shown in III fig.1, the protein was phosphorylated, able to self-oligomerize and bind to SV40 virus large T protein. Moreover, this protein was able to modulate SV40 DNA replication in vitro, bind cellular regulatory protein $p34^{cdc2}$ and be used as the substrate for phosphorylation by $p34^{cdc2}$ (VI). Therefore we concluded that human p53 synthesized in insect cells is by many existing biochemical and biolgical criteria similar to p53 produced in primate cells.

p53 CAN MODULATE REPLICATION FROM SV40 ORIGIN PARTIALLY DUE TO DOWNREGULATION OF SV40 T ANTIGEN HELICASE ACTIVITY (IV)

In this series of experiments we addressed the question: what is the point of action of p53 in cell cycle? As native p53 is located in the cell nucleus, the first obvious points to study were DNA replication and transcription processes. We exploited in vitro DNA replication system (Li and Kelly,1984) using HeLa cell lysates, immunopurified T antigen and p53 (ex-

pressed in either insect or monkey COS cells). For transcription studies we used HeLa cell lysate based eukaryotic transcription system (Handa et al.,1981).

We found (IV fig. 2A) that at least some forms of mouse p53 (dl162) are able to block completely SV40 DNA replication, whereas other forms(dl518) are not. The same was true for human p53. Analysis of SV40 replication products showed that the replication block imposed by p53 must be localized at the initiation of DNA synthesis rather than during elongation or deconcatenation since there is no evidence for preferencial labelling of restriction fragments at or near ori (IV fig.2B).

One possible explanation for the suppression of SV40 DNA replication by p53 is that p53 enhances transcription from plasmid DNA thus changing the equilibrium between transcriptionally active and replicating plasmid molecules, resulting indirectly in inhibition of plasmid replication. In vitro transcription assays (IV fig. 3) showed that presence of p53 had no influence on either amount or ratio of early and late transcripts from SV40 DNA. Thus, we conclude that inhibition of SV40 replication is not based on indirect effects of p53 on transcription regulation.

During lytic infection of SV40, T antigen is the only viral protein required for SV40 minichromosome replication. Therefore we tested whether different forms of p53 act differently in DNA replication through modulating SV40 large T. We found that although p53 only moderately reduces T antigen binding to SV40 origin of replication (IV fig.4) and T antigen ATPase activity is unaffected by p53 (IV fig. 3), it does indeed inhibit T antigen helicase activity to some 65% (IV fig. 6). Although this does not fully explain the role of p53 in DNA replication, we conclude that suppression of SV40 replication by mouse p53 is partially caused by interference with T antigen helicase activity.

DIFFERENT MUTATIONS IN p53 LEAD TO DIFFERENT ACTIVITY IN REPLICATION (V)

As already shown (IV), different forms of p53 (i.e. dl162 and dl518, which are both mutant forms with deletions) act differently in DNA replication assay. To gain a more detailed understanding of the structural requirements for p53-mediated inhibition of SV40 replication, we generated sets of different mouse and human p53 mutants (V fig.2 and fig.3) and they were assayed for DNA replication by transient expression in COS cells (Braithwaite et el.,1987). As shown in V fig.2 and fig.3, different mutants had most different effects in

this assay: some of them inhibited replication fairly well, others did not and some mutants even stimulated replication to certain extent. Quite dramatically, even a single point mutation (i.e. 274Pro-Leu change in cl8 mouse p53, V fig.2) is enough to lead to very big differences in p53 activity.

Correlation of SV40 large T binding ability of mutants and their activity in replication showed that T antigen binding, although necessary, is insufficient for p53-mediated suppression of SV40 DNA replication: there were mutants, which did bind T but were inable to suppress DNA replication (i.e. cl187). It seems that a second distinct activity, unrelated to T-antigen binding, is implicated in suppression of DNA replication by p53.

p53 INTERACTS SEQUENCE-SPECIFICALLY WITH SV40 TRANSCRIPTIONAL ENHANCER (III)

Our findings described above led us to study whether p53 can directly interact with SV40 regions known to control replication. No binding of double-stranded SV40 HindIII-KpnI fragment (ori) was seen on bandshift assay. Single-stranded fragment, however clearly showed interaction with p53 in the same assay (III fig.2). To find more precisely the sequence of SV40 DNA involved in this interaction, we used several methods: a)bandshift assays with different restriction fragments of SV40 (III fig.2 and 3), b)exonuclease assay (III fig.5), c)competition with in vitro synthesized oligonucleotides (III fig.6) and d)in vitro sythesis of hypothetical binding site (III fig.7). Our findings showed that both strands of DNA interact with p53 in single-stranded form and the minimal sequence of SV40 needed for this interaction spans from nucleotides 144 to 214.

p53 INTERACTS WITH CELLULAR REGULATORY PROTEIN $p34^{cdc2}$(VI)

The activities of p53 are likely to impact upon the cell via interactions with other cellular proteins, some of which may be functional analogues of the p53-binding viral gene products (or their competitors). One such candidate was a polypeptide of approximately 34 kD of molecular weight which had been reported to coprecipitate with p53 derived from mouse cell lines (Milner et al.,1989). Our next studies were designed to clarify whether human p53 could be able to interact with eukaryotic cell cycle control protein $p34^{cdc2}$

and be used by the latter as a substrate for phosphorylation.

We carried out cotransfections into COS cells of a mutant human p53 cDNA hudl164 (which is structural homolog of mouse dl162 and reflects the properties of wild-type p53) and human cdc2 DNA. Immunoprecipitation studies of transfected cells showed (VI fig.1a) that a fraction of p53 in these cells indeed coprecipitated together with cdc2 by cdc2-specific antibodies. N-chlorosuccinimide cleavage of coprecipitated protein confirmed that this was protein p53 (VI fig.1b).

We reasoned that the apparent association between p53 and $p34^{cdc2}$ might result from some enzyme/substrate relationship and this was also supported by the presence of $p34^{cdc2}$ kinase recognition motifs in p53 structure. Two model peptides consisting of three linear repeats of the sequence NTSSSPQPY (peptide 1, corresponding to residues 311-318 of p53) or PLSSSVPSY (peptide 2, residues 92-99) were synthesized. We showed that peptide 1 was significantly phosphorylated in the presence of $p34^{cdc2}$ kinase immunoprecipitated by anti-cdc2 antiserum (VI fig.2b).

We also demonstrated that human p53 protein from insect cells could be phosphorylated by $p34^{cdc2}$ (VI fig. 2c). From these results we concluded that p53 is a substrate of the $p34^{cdc2}$ kinase in vitro. Studies with different variants of peptide 1 showed that of three serine residues only one corresponding to Ser-315 of p53 was specifically phosphorylated by $p34^{cdc2}$ kinase.

Studies with cell cycle stage specific extracts from centrifugally elutriated HeLa cells indicated that peptide 1 was utilized as substrate in a cell cycle restricted manner (VI fig.3). We concluded from these data that p53 is a potential $p34^{cdc2}$ kinase substrate from the onset of S-phase. Our observations suggest that phosphorylation of p53 by $p34^{cdc2}$ kinase may regulate the activities of p53 in the initiation step of DNA replication in mammalian cells.

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C. DISCUSSION

I. NEW REAGENTS TO STUDY HUMAN p53

I.1. trpE-p53 FUSION PROTEIN FROM BACTERIAL CELLS IS FUNCTIONALLY INACTIVE BUT SERVES AS A GOOD ANTIGEN FOR p53-SPECIFIC ANTISERA PRODUCTION

Although since late 1970-ies p53 has been quite extensively studied oncoprotein, most of this work was done using murine system.One reason, why investigators were less active in studying human p53 was lack of suitable reagents and, first of all, specific antibodies and purified protein. Before our studies the only source for human-specific p53 antisera was serum from cancer patients - about 10% of cancer patients with different diagnosis have circulating anti-p53 antibodies (Crawford et al.,1982, de Fromentel et al., 1987). Murine-specific monoclonal antibodies pAb421/122 (Gurney et al,1980, Harlow et al.,1981) which cross-reacted with human p53 were not always useful. For example, the epitope for this antibody on p53 (C-terminal amino acid residues 370-378 (Wade-Evans and Jenkins,1985)) could be masked by interactions with other cellular components. Moreover, Xenopus p53 does not contain the amino acids corresponding to pAb421/122 binding site at all (Soussi et al.,1987) and, therefore, can not bind to this antibody.

We constructed recombinant plasmids from which bacterial cells could transcribe-transiate large amounts of fusion protein containing either all amino acid sequences from different species (human, Xenopus) or only some parts of these sequences. Injection of purified fusion proteins into rabbits caused extensive production of anti-p53 antibodies. Although p53 molecules have quite conserved amino acid sequences (81% homology between mouse and human p53, Soussi et al.,1990), our antisera exhibited considerable species specificity. These antisera could be used for broad range of p53 studies.

Functional studies of bacterial p53 showed, however, that it was inactive by many biochemical and biological criteria either because of irreversible denaturation during purification or because of differences in posttranslational modification patterns of eukaryotic cells. To produce functionally active human p53 in large amounts we needed another expression system.

I.2. HUMAN p53 PRODUCED IN INSECT CELLS EXPRESSES BIOCHEMICAL AND BIOLOGICAL PROPERTIES SIMILAR TO p53 PURIFIED FROM PRIMATE CELLS

Baculovirus expression system (Summers and Smith,1987) has been widely used to produce a variety of eukaryotic proteins in insect cells (reviewed in Miller,1988). The foreign protein is usually produced in large amounts and, although there are minor differences in posttranslational modification systems of vertebrate and insect cells, the resulting protein is usually functionally active. Useful characteristics of this expression system are quite simple scaling-up of protein production and mild conditions which can be used for further purification of produced protein. Immunoaffinity purification on monoclonal antibody columns gave human p53 protein, which was highly active by many biochemical (phosphorylation, oligomerization, SV40 large T antigen binding) as well as biological criteria (human p53 produced by insect cells modulated DNA replication, bound to cellular regulatory protein $p34^{cdc2}$ and could serve as a substrate for $p34^{cdc2}$ kinase activity). Therefore we conclude that human p53 protein produced in insect cells can be used as a model substrate for functional studies of p53 in order to gain information about molecular mechanisms of action of this protein.

II. FUNCTIONAL STUDIES OF p53 PROTEIN

II.1. HOW IS p53 EXPRESSED IN DIFFERENT CELLS?

The protein p53 is known to be associated with cellular transformation and tumorigenesis (see Introduction for references). Evidence that p53 itself possesses intrinsic oncogenic potential comes from experiments which show that p53 expression constructs can immortalize cells and can cooperate with an activated ras oncogene in the conversion of such cells to a fully transformed phenotype. A second line of evidence suggests that p53 may act as a tumor suppressor gene ("recessive oncogene"). Such different functions of p53 could be in principle explained by different modulators of p53 function in different stages of cell development.

We undertook a study to investigate how p53 is expressed (on the level of RNA and protein) in differnt established human tumor cell lines in order to find possible abnormalities in p53 expression. We could reveal immunologically abnormal p53 protein in human fibrosarcoma cell line HT 1080. Although these cells contained normal levels of p53 DNA and mRNA, p53 protein had much longer half-life in these cells and its C-terminus was not recognized by monoclonal antibody pAb421, which could reflect an interaction of this part of protein with other cellular component(s).

This was the first indication of possible in vivo interaction of human p53 with cellular components. It was known already that p53 could bind to transforming proteins of DNA tumor viruses (SV40 large T, adenovirus E1B, more recently papillomavirus E6, see INTRODUCTION for references). We also showed that HeLa cells, which contained normal amount of p53 DNA and mRNA, had only very small amounts of protein. Recently it has been suggested that the latter effect is due to rapid degradation of complex HPV E6-p53 (Scheffner et al., 1990). We proposed that such intracellular interactions could explain seemingly contradictory functional activities of oncoprotein p53 in cellular transformation. Such interactions themselves could modulate p53 activity in cells and, therefore, serve as "mechanism for oncogene activation or inactivation". If it is true that functional activity of p53 can be modulated by other proteins, there must be cellular analogs for viral SV40 large T or adenovirus E1B proteins, or competitors with such products for complex formation with p53. One of our further projects was designed to find such cellular proteins.

II.2. WHAT IS THE POINT OF ACTION OF p53 IN CELL CYCLE?

As p53 is normally located in cell nucleus, it could be involved in DNA replication and/or RNA transcription processes. More and more evidence are now accumulating about possible role of p53 in DNA replication . In lyticaliy infected cells T antigen - p53 complexes are found associated with both replicating and mature SV40 DNA (Tack et al.,1986). In in vitro plate-binding assays, mouse p53 can both displace DNA polymerase alpha from complex with T antigen and exist as a trimeric T antigen - pol alpha - p53 complex (Gannon and Lane,1987). Expression of mouse p53 in SV40 permissive monkey COS cells results in a profound inhibition of SV40 origin-dependent DNA replication (Braithwaite et al.,1987).

We exploited in vitro SV40 replication system (Li and Kelly,1984) to study the effects of immunopurified p53 in DNA replication. We were able to show that some forms of p53 are able to completely block SV40 DNA replication in vitro and the blocking occurs in the initiation step of DNA replication (IV and V). At the same time our data showed that p53 has no effect on the regulation in vitro of SV40 early and late transcription by T antigen. Therefore, our data strongly support the suggestion that the primary point of action of p53 is cellular replication. Later our data were confirmed by independent study from another laboratory (Wang et al.,1989).

Recently there have been publications according to which p53 can also act as transcriptional activator (O'Rourke et al.,1990, Fields and Jang,1990, Raycroft et al.,1990). Although these data are somewhat indirect, they may well reflect the fact that the regulation of transcription and DNA replication are in fact connected to each other.

II.3. IS THE ACTIVITY OF p53 TO SUPPRESS SV40 DNA REPLICATION CAUSED BY MODULATING LARGE T FUNCTION?

The only viral protein needed for SV40 lytic infection is large T antigen and all other components of DNA replication are cellular (Kelly, 1988, Tsurimoto et al.,1990). Both mouse and human p53 are known to bind SV40 large T (Lane and Crawford,1979, Linzer and Levine,1979, Sarnow et al.,1982a).Inhibition of SV40 DNA synthesis by p53 correlates well with the ability of p53 to bind T antigen (Braithwaite et al.,1987, IV), indicating that p53 might interfere with the replication process by disturbing normal T antigen functions. Therefore, we undertook an investigation to see whether cellular protein p53 can

change known activities of large T antigen.

Specific binding of large T to the SV40 origin of replication is absolutely required to start the synthesis of each daughter molecule (Deb et al.,1986, Margolskee and Nathans,1984, Paucha et al., 1986, Cole et al.,1986, Li et al.,1986). One possible explanation for the inhibition of SV40 DNA replication by p53 might be that p53 is sequestering T antigen from its binding sites at the origin of replication and thus preventing initiation of DNA synthesis. We found that binding of T antigen to the origin fragment (HindIII-KpnI of SV40) was unaffected by p53 (IV). Although moderate reduction in site II binding could be detected in the presence of vast excess of p53, it is not sufficient to explain its suppression of SV40 DNA replication.

Enzymatic activities of large T required to fulfill its biological functions in SV40 DNA replication are ATPase activity (Clark et al.,1981, Giacherio and Hager,1979) and DNA helicase activity (Stahl et al.,1986, Dean et al.,1987). We showed (IV) that while ATPase activity of T antigen and regulation of SV40 early and late transcripts are unaffected, p53 does moderately inhibit T antigen helicase activity. These findings are not, however, sufficient to explain the underlying mechanisms of suppression of SV40 replication by p53.For example,2 pmoles of mouse dl162 p53 blocks in vitro SV40 DNA replication performed with 5 pmoles of T antigen almost completely. However, the amount of p53 necessary for efficient inhibition of T antigen helicase activity was three to five fold higher. Moreover, analysis of mutant 187p53 protein showed (V), that although this mutant protein binds tightly to T antigen and is exclusively nuclear, it still does not suppress SV40 DNA replication and even enhances it slightly.

Our conclusion from these studies was that, although binding of p53 to T antigen is absolutely needed for suppression of SV40 DNA replication, this process can be only partially caused by interference with T antigen helicase activity. We proposed that other functions of p53, independent of large T binding, contribute to its activity in SV40 DNA replication.

II.4. DOES p53 SUPPRESS SV40 DNA REPLICATION BY INTERACTING WITH ITS DNA SEQUENCES ?

Another way to explain the role of p53 in SV40 DNA replication is to propose some sort of specific interaction between p53 and regulatory regions of SV40 DNA. The most important control region in SV40 DNA lies within its HindIII-KpnI fragment and contains both origin (including three large T binding

sites) and transcriptional enhancers which are exact repeats of 72 base pairs (see Gutierrez et al.,1990). Large T binding site II consists of early palindrome and AT-rich region, which is the primary site for DNA unwinding and the start of DNA replication.

Nonspecific interaction between mouse p53 and double-stranded as well as single-stranded total calf thymus DNA has been described earlier (Steinmeyer and Deppert,1988), but no evidence about sequence-specific binding of p53 to DNA had been published before our studies.

We demonstrated in III that SV40 transcriptional enhancer region contains a DNA stretch which can sequence-specifically interact with human p53 protein. This interaction takes place with single-stranded DNA only, whereas no specific interaction with double-stranded DNA could be detected. Both strands of SV40 DNA could interact with p53. We determined localization of DNA sequence interacting with p53 with several independent methods (III) and found that it involves nucleotides 144-214 on the upper strand. From our experiments it can be seen that the site of p53 recognition is not just the linear sequence of DNA but rather involves secondary or higher structures of DNA.

One seeming contradiction lies between the fact that the site for p53 recognition is located within the transcriptional enhancer and our previous results (IV,V), that p53 is involved in SV40 DNA replication and not in its transcription. It is now known, however, that SV40 enhancer region is required not only for regulation of its transcription, but also for its replication control (see DePamphilis,1988, Kelly,1988) . Moreover, some authors report that there is in fact certain DNA sequence, which is indispensable for both origin and enhancer functions, but additional sequences are needed for maximal origin and enhancer activities (Ariga et al.,1989). We believe that the region of SV40 sequence which we found is able to interact with purified human p53, is at least one site of action of p53 in DNA replication (in addition to modulating T antigen helicase activity as we reported in IV). Our current view is that DNA replication starts with unwinding of DNA in the origin region (by large T in case of SV40 replication with the help of some cellular proteins (Tsurimoto et al.,1990). One role for p53 here is to regulate T antigen helicase activity. Its another (and, probably, structurally different) function is to stabilize single-stranded structure of DNA and thus regulate the formation of replication elongation complex. The latter activity has been shown to be important for SV40 replication and can be replaced by E. coli single-stranded DNA binding protein ssB (Dean et al.,1987, Wold et al.,1987, Dodson et al.,1987). Such a conclusion is in good correlation with our previous finding, that p53 acts in

early stages of SV40 replication (IV, V).

II.5. WHAT CAN BE THE ROLE OF MUTATIONS IN p53 ACTIVITY?

During several recent years there has been a considerable accumulation of data about changes in the structure and expression of p53 gene in most different forms of human tumors (Baker et al.,1989, Nigro et al.,1989, Takahashi et al.,1989, Malkin et al.,1990, Srivastava et al.,1990, Chen and Haas,1990, Bressac et al.,1990, Mulligan et al.,1990, Rodrigues et al.,1990). Very often such changes involve point mutations of p53 gene in certain "hot spots".

Our results clearly demonstrate (V) that a single point mutation in p53 can lead to drastical differences in its activity. Clone 8 of mouse p53 (V) contains a point mutation at amino acid residue 74, leading to proline to leucine change. This change causes significant difference in p53 activity in SV40 DNA replication: clone 8 only slightly suppresses replication whereas wild-type p53 almost completely blocks it.

The same is true for human p53. Our original clone from A431 cells (Harlow et al.,1985) contained single point mutation at amino acid residue 273 causing Arg-His change. Such a mutant form of human p53 did not inhibit SV40 replication (V), whereas wiid-type human p53 was able to suppress SV40 replication as efficiently as mouse wild-type p53 (Stürzbecher et al., IVth International p53 Workshop 1990, Friedman et al.,1990). Moreover, introduction of small insert into p53 (mutant huins615, V) leads to opposite effect: this mutant reproducibely overreplicates more than 2.5-fold compared to control constructs. Two conclusions can be made from these data. First, these data argue strongly for a direct role of p53 in the enzymology of viral DNA replication, since introduction of mutants into regions of p53 protein can cause either inhibition or stimulation of DNA synthesis. Second, as different point mutations can either activate or inactivate different functions of p53, they can form the basis of p53 being an "activated oncogene" or "anti-oncogene". Obviously,this can not be the only reason for tumor progression: very many cancer tissues do not contain any mutations in p53 and the function of p53 can be regulated at the level of intracellular interactions.

II.6. HOW CAN THE ACTIVITY OF p53 BE REGULATED THROUGHOUT THE CELL CYCLE?

The data we present in VI provide evidence that p53 can interact with protein $p34^{cdc2}$ and the latter can phosphorylate amino acid residue Ser-315 in p53 in vitro.

cdc2 was first defined in the fission yeast S. pombe (Nurse,1985, Hayles and Nurse,1986) and genetic data from this experimental system have defined two distinct cell cycle control events which require the cdc2 gene product. The first, called START, is located in the late G_1 and marks the commitment of cells to the mitotic cycle (Nurse and Bissett,1981). The second event is located in late G_2 and marks the initiation of mitosis (Nurse,1975, Thuriaux et al.,1978).

$p34^{cdc2}$ is the mammalian homolog of S. pombe cdc2 (Dunphy et al.,1988). The two proteins share 63% homology and $p34^{cdc2}$ does complement mutations in the cdc2 gene of S. pombe (Lee and Nurse, 1987). In higher eukaryotes there are less data on $p34^{cdc2}$ functions, but a body of evidence implicates $p34^{cdc2}$ in the action of Maturating Promoting Factor (MPF) at the G_2 to M transition (Dunphy et al.,1988) and specifically as the catalytic subunit of the MPF associated Serine/Threonine histone H1 kinase (Arion et al.,1988). One can assume that a possible role for an association between p53 and $p34^{cdc2}$ might be to confer substrate and/or cell cycle position specificity to the $p34^{cdc2}$ associated Ser/Thr kinase.

The human homolog of cdc2, CDC2Hs, has been cloned by marker rescue (Lee and Nurse,1987) and it was able to replace both the G_1 and mitotic functions of the cdc2 gene (Lee and Nurse,1987) as well as the G_1 function of the Saccharomyces cerevisiae homologous gene CDC28 (Wittenberg and Reed,1989). Therefore, it is clear that $p34^{cdc2}$ can fulfil some role at mammalian cell G_1-S transition. Very recently, this idea has gained considerable experimental support (McGowan et al.,1990, D'Urso et al.,1990, Furukawa et al.,1990, Broek et al.,1991).

The use of SV40 DNA replication as a model system has indicated that p53 protein is involved in initiation functions at the onset of DNA replication (III,IV and V) and we have proposed, that, by analogy, p53 might be involved in concert with cellular helicases in the recruitment of cellular replication origins at and beyond the G_1-S boundary. In VI we report that p53 is a substrate for the $p34^{cdc2}$ kinase in vitro and that kinase activity derives from cell populations enriched for S-phase and beyond. The peptide 1 substrate motif (VI) maps to a site on p53 that is phosphorylated in vivo and more

heavily phosphorylated in transformed than in untransformed cells (Samad et al.,1986, Meek and Eckhart,1988).

One possible interpretation of our data suggests a mechanism whereby a replication-related activity of p53 might be controlled during cell cycle (fig.2) In tis model p53 protein accumulating in G_1 would lack phosphate modification at the peptide 1 site and would be either inactive in, or an active suppressor of, origin recruitment. Upon phosphorylation by $p34^{cdc2}$ associated S phase kinase, p53 would actively participate in, or fail to suppress, the initial events of DNA replication, i.e. recognition and/or unwinding of replication origins prior to the onset of strand synthesis. It may well be that phosphorylation of mouse p53 at Ser-389 by casein kinase II (Meek et al.,1990) gives additional variants to such a regulation of p53 activity.

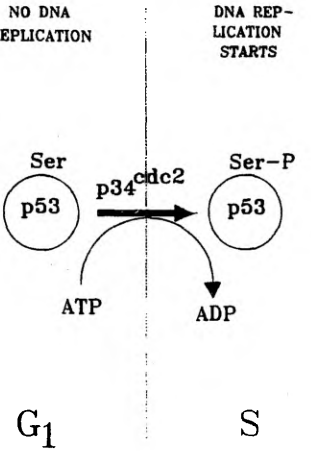

Figure 2 A scheme explaining possible mechanism of controlled action of p53 in cell cycle. Nonphosphorylated p53 is either inactive in, or active suppressor of DNA replication in G_1. p53 phosphorylated by $p34^{cdc2}$ kinase allows DNA replication in S phase of cells.

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Т.О. МАЙМЕТС, Дж.Р. ДЖЕНКИНС

МОДИФИЦИРОВАННЫЙ ОНКОБЕЛОК p53 В ОПУХОЛЕВЫХ КЛЕТКАХ ЛИНИИ HT 1080 ЧЕЛОВЕКА

(Представлено академиком А.А. Баевым 4 III 1987)

Белок p53, кодируемый клеточным онкогеном, является фосфобелком короткого периода распада, который, как правило, локализован в клеточном ядре. Его кажущаяся молекулярная масса по данным гель-электрофореза 53 кД, нуклеотидная последовательность его гена консервирована в эволюции. Хотя функция этого белка в клетках пока неизвестна, многие данные указывают на возможную роль p53 в онкогенезе (для обзора см. [1, 2]): в крови лабораторных животных, а также пациентов со злокачественными опухолями часто циркулируют p53-специфические антитела. Трансформированные вирусами или канцерогенами клетки часто содержат больше белка p53, чем соответствующие нетрансформированные клетки. Белок p53 дает прочный комплекс с большим Т-антигеном вируса SV40 и белком E1B аденовируса, которые являются необходимыми для трансформации клеток этими вирусами. Имеются данные о возможной роли p53 при переходе клеток от состояния покоя в фазу S, а также о роли p53 в трансформации и иммортализации первичных клеток.

В данной работе мы исследовали экспрессию онкобелка p53 в опухолевых клетках линии HT 1080, полученных из фибросаркомы человека. Клетки HT 1080

являются быстроделящимися и полностью трансформированными. Полученные данные мы сравнивали экспрессией p53 в нормальных лейкоцитах человека, а также в малотрансформированных клетках линии меланомы человека RPMI 5966.

В данной работе впервые показано присутствие модифицированного белка p53 в линии опухолевых клеток человека. Хотя точный характер модификации пока неизвестен, можно предположить, что в клетках HT 1080 С-концевой участок молекулы p53 связан с каким-то другим клеточным компонентом, который делает этот участок недоступным к антителам.

В первом этапе работ мы определяли количество белка p53 в клетках линий HT 1080 и RPMI 5966 методом иммунопреципитации [3]. Лизис меченных [^{35}S]метионином клеток проводили в присутствии додецилсульфата натрия и тритона X-100. Белок p53 осаждали суспензией фиксированных клеток Staphylococcus aureus (Immunoprecipitin, BRL) и моноклональными антителами pAb421, узнающими С-концевой участок молекулы p53 [4]. Полученный осадок анализировали методом гель-электрофореза в присутствии додецилсульфата натрия [5] и флюорографии.

Оказалось (рис. 1 см. вкл. между стр. 736–737), полоса *3* и *9*), что моноклональными антителами pAb421 осаждается белок p53 только из клеток RPMI 5966. Такие же были результаты осаждения p53 моноклональными антителами pAb122, участок связывания которых также расположен на С-конце молекулы p53 [4]. Возник вопрос: значит ли этот результат, что: 1) клетки HT 1080 совсем не содержат белка p53; 2) отсутствует участок связывания из-за делеции в гене p53 или 3) участок связывания замаскирован благодаря взаимодействию с каким-то клеточным компонентом?

Для анализа гена p53 был использован метод переноса фрагментированной рестриктазами ДНК на нитроцеллюлозные фильтры по Саузерну и гибридизации фильтров с p53-специфической ДНК [6]. В качестве пробы использован EcoRI–SalI-фрагмент кДНК p53 [7], меченный d[^{32}P]CTP методом ник-трансляции [8]. Как видно на рис. *2а*, (см. вкл. там же), в геноме клеток HT 1080 присутствует ген белка p53. Рестрикционный анализ ферментами BamHI, HindIII, EcoRI и BglII показал, что ген p53 в клетках HT 1080 не содержит больших делеций по сравнению с геном p53 в нормальных лейкоцитах и опубликованной структурой гена p53 фетальной печени человека [9].

Мы также не нашли существенных различий на уровне p53-специфической мРНК (рис. *2б*). Суммарная РНК клеток линий RPMI 5966 и HT 1080 была выделена по описанной методике [10] и очищена на колонках с олиго-dT-целлюлозой. После гельэлектрофореза на агарозе и переноса РНК на нитроцеллюлозный фильтр [11] ее гибридизировали с p53-специфической анти-мРНК, синтезированной SP6-полимеразой в. присутствии [^{32}P]CTP [13]. Как видно на рис. *2б*, количество p53-специфической мРНК в клетках HT 1080 было даже несколько выше, чем в клетках RPMI 5966 или нормальных лейкоцитах. Анализ мРНК с нуклеазой S1 в присутствии клонированной в фаге M13 кДНК p53 [12] также показал, что количество p53-специфической РНК в несколько раз выше, чем в клетках RPMI 5966 или лейкоцитах.

Как упомянуто выше, часто в крови пациентов со злокачественными опухолями циркулируют антитела против белка p53. Так как такие антитела являются поликлональными, они могли бы узнавать разные эпитопы молекулы p53. Как видно на рис. 1, полоса *4–6*, такие сыворотки пациентов, имеющие анти-p53 антитела, действительно осаждали белок p53 из клеток HT 1080.

Таким образом, мы показали, что клетки HT 1080 содержат белок p53, который имеет нормальную структуру на уровне гена и РНК, однако не связыва-

ется с антителами, специфическими к С-концу молекулы. Можно предполагать, что этот участок молекулы недоступен антителам из-за взаимодействия с каким-то другим клеточным компонентом. Известно [14], что связывание белка p53 с большим Т-антителом в трансформированных вирусом SV40 клетках ведет к стабилизации белка, видимо, из-за его недоступности к протеазам. Период полураспада p53 в нетрансформированных клетках около 20 мин, а в трансформированных вирусом SV40 клетках – больше 20 ч.

Мы определяли период полураспада белка p53 в клетках НТ 1080, используя импульсное мечение белков [^{35}S]метионином и последующую инкубацию в присутствии немеченого метионина [3]. Иммунопреципитация p53 из таких клеток анти-p53 специфической сывороткой показала, что белок p53 в клетках НТ-1080 действительно намного стабильнее (период полураспада около 4 ч, рис. 3, см. вкл. между стр. 736–737), чем в клетках RPMI (20–25 мин). Это еще раз говорит в пользу предположения о взаимодействии p53 с какой-то другой молекулой в клетках НТ 1080.

Какие клеточные компоненты могут быть кандидатами такого взаимодействия с белком p53? Если это какой-то белок, можно было бы ожидать его копреципитации с анти-p53-специфической сывороткой. Обращаем внимание на то, что преципитация p53 из НТ 1080 действительно дала две полосы с очень близкими молекулярными массами (рис. 3). Не исключено, конечно, взаимодействие с другими клеточными компонентами (например, нуклеиновыми кислотами).

В последнее время все больше публикуют данные о биполярном действии онкобелков: с одной стороны, они вызывают бесконечный рост недифференцированных клеток и их трансформацию, с другой стороны, они (например, продукты генов fos, ras, src), видимо, связаны с дифференциацией клеток. Такое противоречие можно объяснить взаимодействием между онкобелком и другими вирусными или клеточными белками которые по-разному могут модулировать активность онкобелков. Известно, например, что онкобелок p53 может связываться с вирусными белками (см. выше), а также клеточным белком 68 К, являющимся белком теплового шока [15]. Такая модификация-модуляция онкобелка другими белками может вести к разным эффектам на клеточном уровне и, тем самым, оказаться "механизмом активации" онкогенов.

**Республиканский биоцентр
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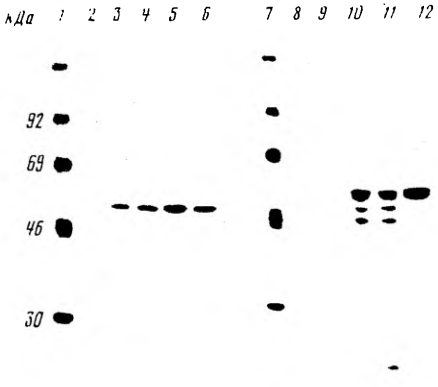

Рис. 1. Иммунопреципитация p53 из клеток RPMI 5966 (полоса *2–6*) и HT 1080 (полоса *8–12*). *1* и *7* – маркеры молекулярной массы; *2* и *8* – преципитация антителами pAb419, неспецифическими к p53; *3, 9* – преципитация антителами pAb421; *4, 10, 5, 11, 6, 12* – преципитация разными p53-специфическими сыворотками

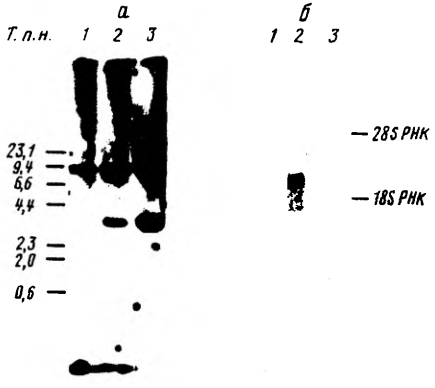

Рис. 2. Анализ геномной p53-ДНК клеток RPMI 5966 (*1*). HT 1080 (*2*) и лейкоцитов (*3*) рестриктазой HindIII по методу Саузерна [6] (*а*) и p53-специфической поли(A)$^+$ РНК этих же клеток [11, см. в тексте] (*б*)

К ст. Т.О. Майметса, Дж.Р. Дженкинса, стр. 757

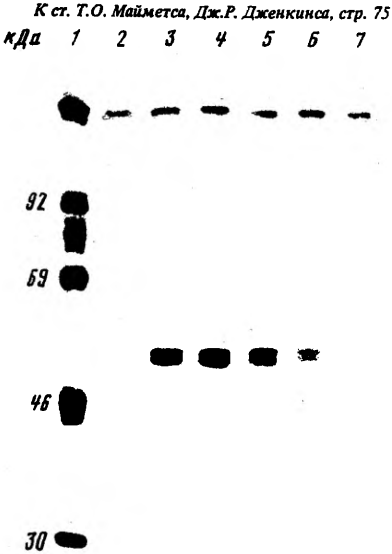

Рис. 3. Анализ времени полураспада белка p53 в клетках HT 1080. Клетки инкубировали [^{35}S]метионином в течение 2 ч, затем продолжали инкубацию в нормальной среде DMEM в течение 0 (*3*), 1 (*4*), 3 (*5*), 5 (*6*) и 7 (*7*) ч. Иммунопреципитацию проводили антителами pAb421 (полоса *3–7*) или pAb419 (*2*). *1* – маркеры молекулярной массы

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Т.О. МАЙМЕТС

БАКТЕРИАЛЬНАЯ ЭКСПРЕССИЯ ЧЕЛОВЕЧЕСКОГО ОНКОГЕНА p53 В ФОРМЕ СЛИТОГО БЕЛКА

(Представлено академиком А.А. Баевым 19 IV 1988)

В последнее время все больше накапливается данных, которые указывают на важную роль клеточного онкобелка p53 как в нормальном развитии клеток, так и в онкогенезе (см. [1] для обзора). Хотя молекулярный механизм действия p53 в клетках неизвестен, имеются многие работы о физико-химических, иммунологических и биологических свойствах этого белка. Абсолютное большинство таких данных, однако, получено по белку p53 мышей. Хотя p53 сравнительно консервативный в эволюции (сродство нуклеотидных последовательностей гена p53 человека и мыши в кодирующем участке 81%, сродство аминокислотных остатков 78% [1, 2]), имеются данные о функциональном различии p53 разных видов. Например, p53 приматов гораздо слабее связывается с большим Т-антигеном вируса SV40, чем p53 мышей [3]. Большинство моноклональных антител, специфических к белку p53 мышей, не связывается с человеческим белком p53 [4]. Экспрессия p53 мышей в клетках Cos1 селективно ингибирует репликацию ДНК от начала репликации SV40, человеческий p53 этого не делает [5].

Количество данных о свойствах белка p53 человека ограничено из-за следующих причин 1. Геномная [6, 7] и кДНК [2] p53 человека клонированы сравнительно недавно. 2. Недостаток панели моноклональных антител, узнающих p53 человека. Для исследования человеческого p53 в данный момент используют лишь 2 мышиных антитела: pAb122 [8] и pAb421 [9], связывающихся с С-концевым участком p53, который, однако, может быть замаскирован благодаря взаимодействию с другими клеточными компонентами [10]. 3. До сих пор не опубликовано работ по выделению чистого онкобелка p53. Эта задача затруднена из-за очень маленького содержания p53 даже в трансформированных клетках, а также из-за образования прочных комплексов между p53 и клеточными и вирусными белками. Очевидно, что продукция p53 (или его фрагментов, представляющих какие-то функциональные домены) в большом количестве дает возможность подробнее охарактеризовать этот белок, а также использовать его в модельных функциональных экспериментах (контроль репликации, транскрипции и т.д.).

В данной работе описывают бактериальную экспрессию человеческого онкогена p53 в форме слитого белка и характеризуют свойства новообразованного белка.

Схема конструирования плазмиды бактериальной экспрессии человеческого p53 pATHhp53 изображена на рис. 1. Плазмида pR4-2 [2] включает EcoRI–SalI-фрагмент кДНК p53 человека (1,76 т.п.н.). Из этой плазмиды был вырезан фрагмент BalI–SmaI длиной 0,84 т.п.н., включающий кодоны 161–393 белка p53 (hp53).

В качестве вектора экспрессии использована плазмида pATHll из семейства векторов pATH (получены от др-а Кэрнера, Колумбийский университет, США). Эта плазмида включает следующие нуклеотидные последовательности: 1) триптофановый промотор (P), супрессируемый триптофаном и индуцируемый индолпропиониловой или индолакриловой кислотами; 2) фрагмент гена антранилатсинтетазы (trpE), содержащий N-концевые кодоны 1–336 этого белка; 3) полилинкерную последовательность (PL) для клонирования вставок в нужной рамке считывания мРНК и 4) последовательности pBR322, включая маркер устойчивости к ампициллину (amp).

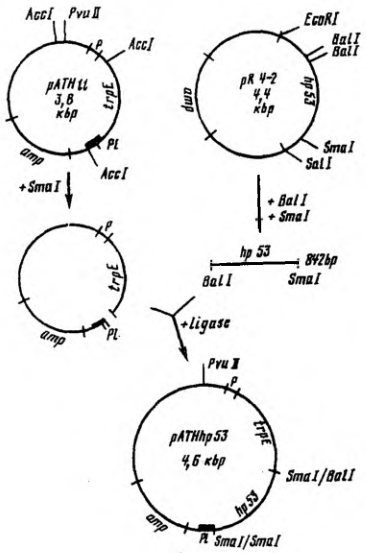

Рис. 1. Схема конструирования плазмиды бактериальной экспрессии p53 человека. Объяснения см. в тексте

Вектор pATH11 был разрезан рестриктазой SmaI. После очистки вектора и 0,84 т.н.п. вставки они были соединены по протоколу лигирования нелипких рестрикционных концов [11] и введены в клетки E.coli RR1 [12]. Выращивание клеток проведено в среде M_9 в присутствии аминокислот, ампициллина и триптофана. В результате образовались колонии бактерий, устойчивые к ампициллину и содержащие последовательность p53 в двух ориентациях. После выделения плазмидной ДНК эти две ориентации p53 были различены рестриктазой AccI: плазмида pATH11 включает 3 участка узнавания для AccI, BalI–SmaI-фрагмент p53 – еще 1 участок. В результате плазмида pATH11hp53 со вставкой в "кодируемой" ориентации дает фрагменты 2,1; 1,5; 0,7 и 0,3 т.п.н., а плазмида со вставкой в "некодируемой" ориентации 2,1; 1,0; 0,7 и 0,7 т.п.н.

Полученные клоны были выращены без добавления в среду триптофана и синтез слитого белка от триптофанового промотора был индуцирован индолпропиониловой кислотой (20 мг/л). После лизиса клеток с 0,6% NP40 в присутствии 0,3 *M* NaCl и лизоцима была выделена мембранная фракция клеток, белковый состав которой проанализирован полиакриламид-гель-электрофорезом в присутствии додецилсульфата натрия [13].

Как показано на рис. 1 (полосы *3* и *4*), клоны 10 и 15, содержащие вставки p53 в плазмидах pATH11 в кодируемой ориентации, синтезировали большое количество белка молекулярной массой около 60 кДа, которая соответствует и теоретической молекулярной массе слитого белка trpE-hp53. В то же время клоны, содержащие плазмиды pATH11 (рис. 2 полоса *1* см. вкл. между стр. 1504–1505) или pATH11hp53 со вставкой в некодируемой ориентации (полоса *2*), такого продукта не синтезировали.

Дальше было исследовано связывание полученного слитого белка с p53-специфическими моноклональными антителами pAb421. Белки были разделены электрофоретически, перенесены на нитроцеллюлозный фильтр и связывание с антителами pAb421 было выявлено иммунопероксидазным методом [4]. Как видно из рис. 2 (полосы *5–8*), только 60-килодальтонные белковые продукты клонов 10 (полоса *7*) и 15 (полоса *8*) связываются с pAb421, в то время как белки клонов с плазмидами pATH11 (полоса *5*) или pATH11hp53 со вставкой p53 в некодируемой ориентации (полоса *6*) с этими антителами не связывались. Следовательно, в сконструированных клонах E.coli (клоны 10 и 15) действительно синтезируется белок, включающий С-концевые последовательности человеческого p53. В тех же условиях слитый белок не связывался с моноклональными антителами pAb607, специфическими только p53 грызунов. Это указывает на сохраненную видоспецифичность слитого белка trpE-hp53.

Слитый белок был очищен из мембранной фракции клона 15 методом гельфильтрации на колонке Superose 6 ("Pharmacia Fine Chemicals) в 20 м*M* tris-HCl-буфере, pH8,0, содержащем 10 м*M* 2-меркаптоэтанол и 0,1% додецилсульфат натрия. Анализ хроматограммы показал, что количество 60-килодальтонного слитого белка в мембранной фракции клеток составляло 35–40% от всего белкового материала. Электрофорезом и иммунопероксидазным методами было показано, что одноэтапная очистка мембранной фракции бактерий дает препарат слитого белка с довольно высокой чистотой (рис. 3*а*, полоса *2*), который включает последовательности p53, связывающиеся с моноклональными антителами pAb421 (рис. 3*б*, полоса *2* см. вкл. между стр. 1504–1505).

Было доказано и присутствие последовательностей белка trpE в выделенном слитом белке (рис. 3*в*). На полиакриламидный гель наносили мембранные фракции клона 15, содержащего плазмиду pATHhp53 (полоса *4*), бактерий E.coli RR1 без плазмиды (полоса *3*) или с плазмидой pATHll (полоса *2*), а также очищенный гель-фильтрацией белок trpE-hp53 (полоса *1*). После электрофореза эти белки были перенесены на нитроцеллюлозный фильтр и связаны с моноклональными антителами D6, специфическими к белку trpE. Белки клеток E.coli RR1 не содержат последовательностей белка trpE, и антитела D6 их не узнавали (рис. 3*в*, полоса *3*), в то время как продукты плазмид pATHll (полоса *2*) и pATHllhp53 (полосы *1* и *4*) связывались с trpE-специфическими антителами. Широкий диапазон распределения по молекулярной массе trpE-специфических последовательностей объясняется, по-видимому, высокой частотой абортивного прекращения считывания trpE.

Таким образом был сконструирован вектор бактериальной экспрессии, продуктом которого является слитый белок, включающий 336 N-концевых аминокислотных остатков белка trpE и 232 C-концевых остатка человеского белка p53. Показано, что очищенный белок связывается с p53-специфическими и trpE-специфическими моноклональными антителами. Слитый белок trpE-hp53 синтезируется в большом количестве и может быть использован для изготовления панели моноклональных антител к человеческому онкобелку p53. В то же время впервые получен чистый человеческий p53-антиген, который отражает некоторые специфические свойства целостного белка и который может быть использован в модельных экспериментах для изучения молекулярного механизма действия онкобелка p53.

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Республиканский биоцентр по генной и клеточной инженерии при Академии наук ЭССР, Тарту

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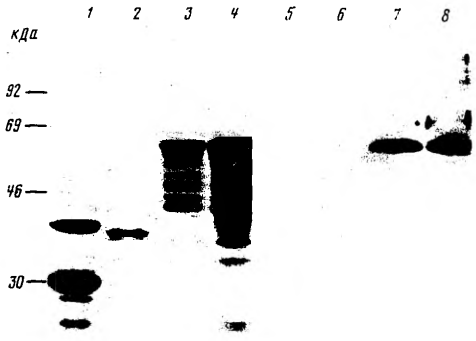

Рис. 2. Анализ белков мембранных фракций разных клонов методом гель-электрофореза в присутствии додецилсульфата натрия (полосы *1–4*) и анализ связывания белков моноклональными антителами pAb421 методом иммуноблоттинга (полосы *5–8*). *1, 5* – клоны, содержащие плазмиды pATHll ; *2, 6* – клоны, содержащие плазмиды pATHllhp53 со вставкой p53 в некодируемой ориентации; *3* и *7* – клон 10; *4, 8* – клон 15

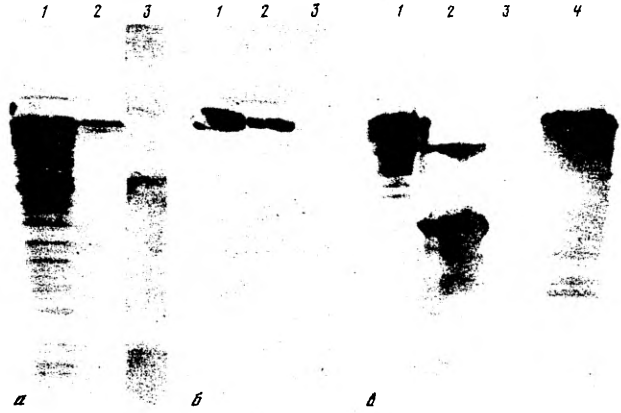

Рис. 3. Анализ очищенного слитого белка trpE-hp53. *а* – полиакриламид-гель-электрофорез в присутствии додецилсульфата натрия мембранной фракции клона (*1*) и очищенного гель-фильтрацией слитого белка (*2*). На полосе *3* – маркеры молекулярной массы 66, 45, 35 и 29. *б* – фильтр иммуноблоттинга, обработанный p53-специфическими моноклональными антителами pAB421: *1* – мембранная фракция клона 15; *2* – очищенный гель-фильтрацией слитый белок; *3* – мембранная фракция клона, содержащего плазмиду pATHllhp53 со вставкой p53 в некодируемой ориентации. *в* – фильтр иммуноблоттинга, обработанный trpE-специфическими моноклональными антителами D6: *1* – очищенный слитый белок; *2* – мембранная фракция E. coli RR6 с плазмидой pATHll; *3* – мембранная фракция E. coli RR1; *4* – мембранная фракция клона 15

THE INTERACTION OF SV40 TRANSCRIPTION ENHANCER WITH HUMAN ONCOPROTEIN p53.

Toivo Maimets(1,2), Horst-Werner Stürzbecher(2), Ruth Brain(2), Will Court(2) and John R. Jenkins (2).
(1)Estonian Biocenter,Tähetorn,Toomemägi, 202400 Tartu,ESTONIA USSR and (2)Marie Curie Memorial Foundation Research Institute,The Chart,Oxted,Surrey,RH8 OTL,UK.

I N T R O D U C T I O N

SV40 has been a remarkably good model to study the events occuring in single replicons (see Kelly, 1988 for review). The viral genome consists of about 5000 base pairs containing an origin of DNA replication. Since SV40 encodes only one replication protein (T antigen), the virus makes extensive use of the cellular replication machinery. Therefore, SV40 replication is a good system to study the role of cellular proteins in DNA replication.

p53, the protein product of oncogene p53, is a cellular oncoprotein which has been shown to be involved in DNA replication (see Sturzbecher and Jenkins, 1988, Soussi et al.,1990 for review). This is a nuclear phosphoprotein which is able to form complex with SV40 T antigen (Lane and Crawford, 1979) as well as with E1b 58 Kd protein of adenovirus(Sarnow et al,1982). p53 can also form complexes with itself and some mutant p53 proteins bind the heat-shock related proteins hsp 72/73 (Pinhasi-Kimhi et al., 1986, Hinds et al., 1987, Sturzbecher et al., 1987).

Recently we have demonstrated that protein p53 can modulate DNA replication (Sturzbecher et al., 1988). To understand possible molecular mechanism of this activity we produced human p53 in insect cells and studied its interaction with SV40 DNA. Here we demonstrate that SV40 DNA interacts sequence-specifically with protein p53 and the binding site lies within the enhancer region of SV40. The interaction takes place with single-stranded DNA only, whereas corresponding region of double-stranded DNA does not interact with p53.As it has been shown (DeLucia et al.,

1986, see also DePamphilis 1988), the enhancer region of SV40 plays active role not only in transcription but also in DNA replication. Therefore we believe that this interaction forms the molecular basis for p53 activity in the regulation of SV40 replication.

METHODS

Construction of recombinant viruses. Construction of recombinant virus from AcNPV by substituting polyhedrin gene to human p53 cDNA was done by procedures described by Summers and Smith (Summers and Smith, 1987) with minor modifications. To clone human p53 cDNA into replacement vector pAcYM1 we designed oligonucleotides which corresponded to 5' and 3' ends of upper and lower strand of human p53 cDNA (Harlow et al., 1985) respectively and contained additional BamHI restriction sites. Using polymerase chain reaction (Scharf et al., 1986) we got human p53 cDNA from Harlow's clone pR4-2 with BamHI ends and cloned it into single BamHI cloning site of vector pAcYM1. Cotransfection of replacement vector and wild-type virus as well as screening of recombinant vector was done exactly by Summers and Smith (Summers and Smith, 1987).

Purification of human p53 from insect cells. Cells (200 ml) were infected with recombinant virus and were grown for further 48 hrs. Cells were collected by centrifugation, lysed in Lane buffer B (Simanis and Lane, 1985) in the presence of PMSF, aproteinin and vanadyl ribonucleoside complex and cleared by ultracentrifugation. The supernantant was precleared by passing it through ProteinA-Sepharose column and subsequently circulated through pAb122-ProteinA-Sepharose column (prepared as described in Wade-Evans and Jenkins,1985). Nonbound material was washed out with Lane buffer B+0,5% NP40 and after that buffer D (Simanis and Lane 1985), then the beads were removed from column and washed with NET-Gel buffer(Jenkins et al., 1984). Bound p53 was eluted with 5 mg peptide Lys-Gly-Gln-Ser-Thr-Ser-Arg-His-Lys-Lys corresponding to the binding site of p53 to monoclonal antibody pAb122. Protein was purified from peptide by passing it through Sephadex G-25 column in Lane buffer F (Simanis and Lane,1985). Phosphorus-32 and sulfur-35 labeling of proteins as well as protein oligomerization assay were done as described previously (Sturzbecher et al., 1987).

Bandshift assay. p53 complex with DNA was formed by mixing DNA in 25 mM ATP, 65 mM creatine phosphate, 0.005 mg/ml creatine kinase, 0.1 mg/ml BSA and 0.2 mg/ml poly(dIdC) in buffer 120 mM HEPES-KOH pH 7.5, 2mM DTT, 15mM magnesium chloride and 15% glyce-

rol with equal volume of protein p53 in buffer F or with buffer F only. To get single-stranded DNA the corresponding restriction fragment of SV40 DNA was boiled for 2 min. in.10 mM EDTA and then kept on ice before adding to reaction mix. The mixture was incubated at 37 degrees and loaded onto 8% native acrylamide-bisacrylamide (44:0.8) gel in TBE buffer . The gel was dried and autoradiographed at -70 degrees.

Exonuclease assay. FokI-KpnI restriction fragment was labeled with T4 Polynucleotide kinase exactly by the procedure described by Maniatis et al (Maniatis et al., 1982) and mixed with p53 complex mixture (see above) so that the volume was 0.015 mls. Equal volume of p53 in buffer F or buffer F alone was added and the complex was allowed to form at 37 degrees for 1hr. Then 0.5 units of T4 DNA Polymerase was added , the reaction was proceeded for 2, 15 and 45 mins and stopped by addition of equal volume of phenol/chloroform mix (1:1). After precipitation with ethanol the reaction products were loaded onto 6% Maxam-Gilbert sequencing urea-polyacrylamide gel. Maxam-Gilbert G+A reaction of FokI-KpnI fragment was used to generate DNA sequence markers.

Competition assay. Different oligodeoxynucleotides corresponding to various sequences of SV40 origin-enhancer region were synthesized on Applied Biosystems DNA Synthesizer and purified by the procedure suggested by manufacturer. The oligodeoxynucleotides were added to FokI-KpnI fragment of SV40 before mixing it with protein in 500-fold molar excess. The complex was formed and analysed by bandshift assay as described above.

All genetic engineering techniques (DNA restriction, ligation and labeling with Klenow fragment of DNA polymerase etc.) were done by Maniatis et al (Maniatis et al., 1982). SDS-polyacrylamide gels were run by the method of Laemmli (Laemmli 1970).

RESULTS

Human p53 synthesized in insect cells. To study the properties of human oncoprotein p53, we constructed recombinant baculovirus by the procedures described by Summers and Smith (Summers and Smith, 1987). Basically, p53 cDNA from Harlow's clone pR4-2 was cloned into vector pAcYM1 (Matsuura et al., 1987) and the construct was cotransfected into Spodoptera frugiperda insect cells together with baculovirus AcNPV. The resulting recombinant virus was purified and when new insect cells were infected with this virus, the cells started to produce human oncoprotein p53.The protein was immunopurified on monoclonal antibody pAb421-proteinA Sepharose column and eluted with peptide, containing the entire binding site of this antibody.

Our first question was whether such a protein is biochemically identical to the one produced by primate cells. As shown on fig.1, the protein was phosphorylated and able to oligomerize and bind to SV40 virus protein large T. Moreover, p53 made in insect cells was able to modulate DNA replication as the protein made in monkey Cos1 cells (Sturzbecher et al., 1988, data not shown). Therefore we concluded that human p53 synthesized in insect cells is by existing biochemical criteria similar to p53 made in primate cells.

Some restriction fragments of SV40 origin-enhancer region bind human p53 in single-stranded form. To test the ability of p53 to bind to certain regulatory sequences involved in DNA replication and/or transcription regulation we prepared several restriction fragments of SV40 HindIII-KpnI region(ori). As a control, a nonrelevant piece of DNA (about 300 bp HincII-PstI fragment of vector CMVp53(Jenkins et al., 1989)) was used. As shown in fig.2, neither ori nor control fragment bound DNA when tested by band retardation assay. However, generating single-stranded DNA by boiling the DNA before binding assay changed the picture. While control DNA was still unable to bind to p53(Fig.2), the ori fragment gave a bandshift. Multiple bands reflect, we think, the fact that p53 itself is able to oligomerize. Principally the same picture was got when we used in vitro synthesized p53 instead of baculovirus protein (data not shown).

To get better localization of binding site, we generated

more fragments from ori fragment and assayed their ability to bind p53. We found , that the binding site lies in FokI-KpnI region of ori fragment, as this was the shortest one which gave bandshift when incubated with p53(see also Fig.4 a). Therefore, one can tell from data obtained from band retardation assay using different restriction fragments of SV40 DNA that SV40 nucleotides 94-298 contain binding site for human oncoprotein p53.

Both strands of SV40 ori fragment bind p53. To see whether only one strand or both of them bind p53, we used different labeling of strands. First, FokI-KpnI fragment of SV40 was labeled with Klenow fragment of DNA polymerase I and subsequently cut with restriction enzyme DdeI. After that procedure, only one ("lower") strand of DNA should be labeled and, as shown in Fig.3, it does bind p53. Then another batch of FokI-KpnI fragment was labeled with T4 polynucleotide kinase, which preferentially incorporates label into 5'-end of "upper" strand, as the 5'-end of the lower one is masked by 3' overhang of KpnI site. As shown in Fig.3, such a fragment binds p53 . Further evidence of p53 binding to "upper" strand came from using synthetic oligodeoxyribonucleotides (see further).

72-bp repeats <u>per se</u> are not sufficient to bind p53. The FokI-KpnI region of SV40 consists of mostly two 72 bp exact repeats. Therefore it was logical to expect that one of these repeats should be sufficient to bind p53. To test this, we made synthetic oligonucleotides of 72 nucleotides, which correspondec to repeated sequences on both DNA strands and checked their ability to bind p53. As it is shown in Fig.4 A, they were not sufficient to form p53 binding site, although they can contain a part of it.

Exonuclease analysis of p53 binding site. To get more detailed picture of p53 binding site on SV40 DNA, we tried to determine the 3'-end of binding site using the idea that bound protein should protect DNA from activity of exonucleases. For that purpose we labeled the 5'-end of FokI-KpnI fragment(upper strand), formed a complex with p53 and treated it with T4 DNA Polymerase, which has got 3'-5' exonuclease activity. As shown in Fig.5, the exonuclease indeed stops in two places in the presence of p53 : at nucleotides 214 and around nucleotides 161-172. From these data one could expect that there are in fact two binding

sites on DNA and one of them is not strong enough to give a bandshift (as synthetic 72-mers themselves did not give any bandshift (see Fig.4, lanes b and c).

Competition experiments. To get independent information about the exact localization of p53 binding site(s), we synthesized a set of various oligodeoxynucleotides corresponding to different regions of SV40 DNA FokI-KpnI fragment and tested their ability to compete for p53 binding when added in vast molar excess. As it is shown in Fig.6, these oligonucleotides fall into three subgroups: first these which completely inhibit ori-p53 complex formation and therefore contain all sequences for this binding or, alternatively, change the overall structure of DNA fragment. Second, there are oligonucleotides which do not change the ori-p53 formation even when added in vast molar excess and therefore do not contain sequences involved in complex formation (or contain only very few of them). And third there are sequences which can inhibit complex formation only partly and therefore seem to contain part of p53 binding site.

The first interesting notion came from the fact that oligonucleotide containing 72-mer repeat of SV40 enhancer (nucleotides 107-178 and 179-250) entirely blocks FokI-KpnI fragment binding to p53 in these conditions (Fig.6 lane D) . That clearly demonstrates that this sequence contains all the nucleotides needed for p53 binding. However, at the same time oligonucleotide 107-178/179-250 itself does not bind p53 (Fig.4). We conclude that this may reflect certain higher structure of binding site, which is formed by parts of both 72-mers when connected into one DNA stretch. This is well consistent with our previous data about exonuclease treatment of the complex (see above). Analysis of data from competition assay allows to conclude that p53 binding site on upper strant lies somwhere between nucleotides 150 and 285 whereas the binding site seems to contain two domains (see also the results of exonuclease assay above): one of them lies in the region of nucleotides 161-173 as nucleotides 161-167 and 165-173 partially competed for complex formation in 500-fold excess (Fig.6 lanes E and F). This is consistent with our data from exonuclease assay: exonuclease had stops at nucleotides 161-173 (Fig.5). Another site (and probably a weaker one) lies further downstream as nucleotide 179-217

inhibited partly the binding (Fig.6 lane J) and region 75-146 is not involved in the binding at all (lane A, see also further).

From the data obtained from experiments above we concluded that SV40 DNA upper strand sequence 144-214 should contain nucleotides which are sufficient to bind human p53 as strongly as FokI-KpnI fragment of SV40. To test this we synthesized oligodeoxyribonucleotide, containing SV40 sequences 144-214 and 164-214(upper strand). As shown in Fig.7, lane b , the first oligonucleotide is able to bind p53 in bandshift assay, whereas the second one is not (lane c). This clearly demonstrates that our proposal about p53 binding site was correct and indeed the site covers parts of both 72-bp repeats of SV40 enhancer. One possible secondary structure created by computer analysis is presented in Fig.8 together with our data about exonuclease stops and competing/noncompeting sequences. Additional data about DNaseI hypersensitive sites of this fragment when complexed with p53 is shown. Unfortunately, "fingerprint" analysis with DNaseI did not give publishable results because of high "background" of single-stranded DNA digestion.

Controls. The specificity of p53 interaction to SV40 DNA is determined by the finding that the binding occurs in the presence of large excess of nonspecific DNA poly(dIdC) and cold specific oligonucleotides compete for this binding when added in molar excess (Fig.6). To demonstrate that the bandshift effect of FokI-KpnI fragment is really due to protein p53, we first treated the complex with proteinase K before loading it onto gel and could demonstrate the loss of bandshift (Fig.4 B). In addition, we added pAb122-Sepharose to the complex, which bound to p53 and coprecipitated the labeled DNA fragment as well (Fig.4 C). However, one should mention the theoretical possibility that there is another component in insect cells which is copurified with p53 and binds to DNA. Although we cannot see any protein contamination of that amount on SDS-gels, we cannot entirely exclude this possibility. The fact is, however, that p53 is able to interact with regulatory region of SV40 transcription regulation, either by itself or through something else.

D I S C U S S I O N

Our previous data have demonstrated that oncoprotein p53 can modulate DNA replication *in vitro* (Sturzbecher et al., 1988)and *in vivo* (Braithwaite et al.,1987). At the same time we found that this activity of p53 is different from its large T antigen binding activity, since there were mutants which did bind large T, but were still unable to change the speed of DNA replication. One way to explain the molecular mechanism of p53 action in replication is to propose that p53 interacts with other cellular proteins which in turn regulate the speed of DNA replication. Such interactions are known for some viral and cellular proteins (large T of SV40, adenovirus E1b, human papillomavirus E6, hsp), although the meaning of these interactions to replication (if there is any) is still obscure. Another way to explain the role of p53 in replication is to propose some sort of specific interaction between p53 and regulatory regions of DNA. There has been a report about nonspecific interaction of mouse p53 with double-stranded DNA as well as with single-staranded DNA (total calf thymus DNA(Steinmeyer and Deppert, 1988)), but there is no good evidence about sequence-specific binding of p53 to single-stranded DNA so far.

In this paper we have shown that SV40 transcription enhancer region contains a DNA sequence which can specifically interact with human oncoprotein p53. The complex is formed with single-stranded DNA only whereas double-stranded DNA does not bind p53 in these conditions. We have determined localization of p53 binding site with several independent methods (band retardation assay with labeled restriction fragments of SV40, exonuclease stop assay, competition experiments with cold synthetic oligonucleotides, bandshift assay with synthetic oligonucleotides) and found that it involves nucleotides 94-298 on both strands of SV40 and more particularily nucleotides 144-214 at least on the upper strand. It can be seen from our experiments that the binding site is not just linear sequence of DNA, but it rather involves some sort of secondary or even higher structure. One can see from computer modelling (Fig.8) that the DNA sequence of SV40 in this region can form stem structures. We believe that these structures bind to p53 and that explains why double-

stranded DNA is not able for such an interaction: base-pairing between two strands is too strong to allow intrastrand structure formation The idea about secondary structure of p53 binding site can also explain the fact that both strands of DNA are able to bind protein: although their sequences are very much different, the stem structures are the same.

One seeming contradiction lies between the fact that the site of p53 binding lies in SV40 transcription enhancer region and our previous finding (Sturzbecher et al., 1988) that p53 is involved in SV40 DNA replication and not in its transcription. It is now known that SV40 enhancer region is needed not only for transcription of its genes, but it also regulates its replication (see DePamphilis, 1988 and Kelly,1988). It has also been shown that there is a c-myc protein binding site in human DNA which has transcriptional enhancer activity as well as autonomously replicating activity(Iguchi-Ariga et al., 1988a). Moreover, the same authors report that there is in fact certain DNA sequence, which is indispensable for both origin and enhancer functions, but additional sequences are needed for maximal origin and enhancer activities (Ariga et al., 1989). Therefore we believe that enhancer-origin regions are very much functionally and structurally combined and connected in DNA sequence. This should mean that the region of SV40 sequence which we find is able to interact with purified human p53, is at least one site of action of p53 in DNA replication process. Our current view is that DNA replication starts with unwinding of DNA in the origin region (by large T in case of SV40 replication and probably some cellular proteins as well (Tsurimoto et al., 1990)). One possible role for p53 after that has occured is to stabilize single-stranded structure of DNA. Such an activity has been shown to be important for SV40 replication and can be replaced by E.coli single-stranded DNA binding protein ssB (Dean et al., 1987, Wold et al., 1987, Dodson et al., 1987).

This single-stranded DNA binding function of p53 can be independent from its activity to bind large T antigen and to modulate its biochemical activities (enhancement of large T ATPase, DNA binding and helicase activity (Sturzbecher et al., 1988, Wang et al., 1989, Tack et al., 1989)).It is known that in large T-producing cells (Cos1, for example) p53 exists always in

two forms: P53 complexed with T and free p53. It seems that this type of DNA binding could be the function of free p53.

There is a report that certain DNA sequences which have origin activity are able to bind p53 in double-stranded form (Iguchi-Ariga et al., 1988b). However, the sequence described by the authors did not bind our purified human p53 neither in single-stranded nor in double-stranded form (not shown). Also, no homology between their sequence and our p53-binding sequence was found by computer analysis. It may be assumed that the method used by them (immunoprecipitation of p53-DNA complex from sophisticated mixture of p53-containing cell lysate and HindIII-digested total DNA) picks up not only p53-binding sequences but also those which bind to other p53-binding cellular components. In addition, by their method no single-stranded DNA binding could be detected anyway.

LEGENDS TO FIGURES

Figure 1. Human p53 protein produced in insect cells is phosphotylated(A), able to self-oligomerize(B) and bind to SV40 large T antigen(C). A: Insect cells producing human p53 were labelled either with ^{35}S-Methionine or ^{32}P phosphoric acid and corresponding lysates were immunoprecipitated with monoclonal antibody pAb421 (421) or normal rabbit serum (NRS) as control. B: ^{35}S-Met labelled lysates were loaded onto sucrose gradients, centrifuged and fractionated (Sturzbecher et al., 1987). Human p53 was immunoprecipitated from different fractions with pAb421 and loaded onto SDS-denaturing gel. The positions of sedimentation markers are shown. C: Human p53 from insect cells was mixed with SV40 large T antigen and immunoprecipitated sequencially three times with pAb421 (lanes1-3). After that the remaining lysate was treated with anti-T monoclonal antibody pAb419 (lane4). The same was done with pAb419 (three times precipitation, lanes 5-7) and then with pAb421 (lane 8). All the precipitations were analyzed on SDS-denaturing gel.

Figure 2. Single-stranded fragments of SV40 ori bind p53. Purified p53 was mixed with either double-stranded DNA (ds DNA) or single-stranded DNA in the conditions described (see Materials and Methods). Bandshift assay was performed using 8% native acrylamide-bisacrylamide gel. Corresponding restriction fragments of SV40 are shown on the diagram above.

Figure 3. Both strands of SV40 bind p53. Fok-Kpn restriction fragment was labelled either from upper strand with T4 polynucleotide kinase (lanes 1 and 2) or from lower strand with Klenow fragment of DNA PolI and subsequently cut with DdeI (lanes 3 and 4). After that bandshift assay was done without(-) and with(+) purified human p53.

Figure 4. A: Neither 72 base pairs upper strand (b) nor lower strand (c) bound p53 in the conditions, where Fok-Kpn fragment did (a). B: Before loading onto the gel the complex Fok-Kpn - p53 was treated with proteinase K. C: Treatment of the complex with pAb122-Sepharose before loading onto the gel.

Figure 5. Exonuclease analysis of p53 binding site on Fok-Kpn fragment. 5'-labelled upper strand was mixed with 200 ng p53 and the complex was treated with 0.4 units of T4 polymerase for different time periods as shown. The resulting products were analysed on denaturing polyacrylamide-urea gel. The positions of molecular weight markers are shown.

Figure 6. Competition experiments with different synthetic oligonucleotides corresponding to the nucleotides shown. 500-times excess of corresponding oligonucleotides were added to the Fok-Kpn - p53 complexes before loading onto native gel.

Figure 7. Synthetic oligonucleotide 144-214 binds p53 (b) as Fok-Kpn control fragment (a), whereas oligonucleotide 164-214 does not (c).

Figure 8. Schematic representation of p53 binding site on SV40 DNA.

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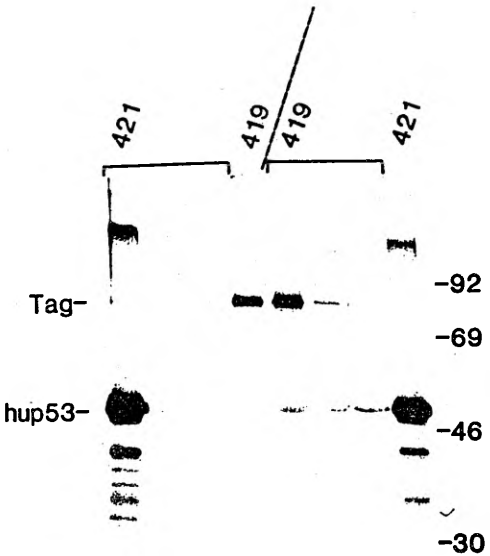
FIGURE 1 AB



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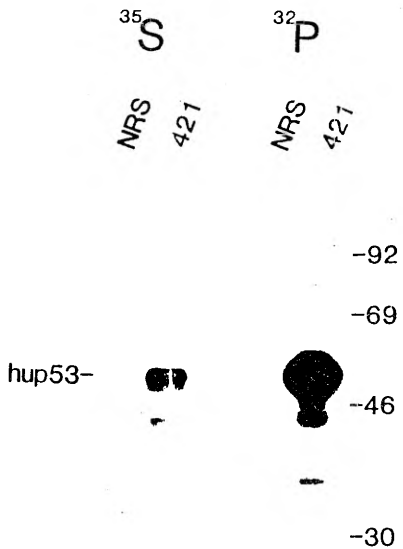
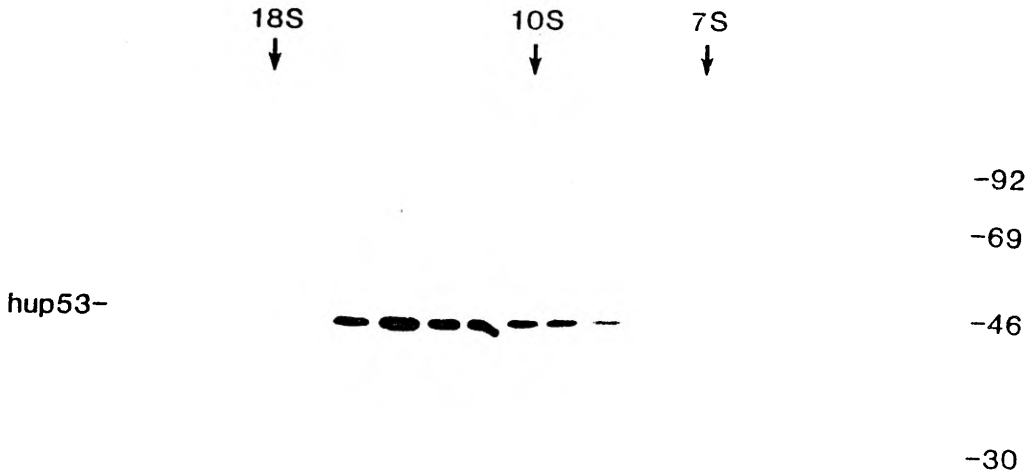


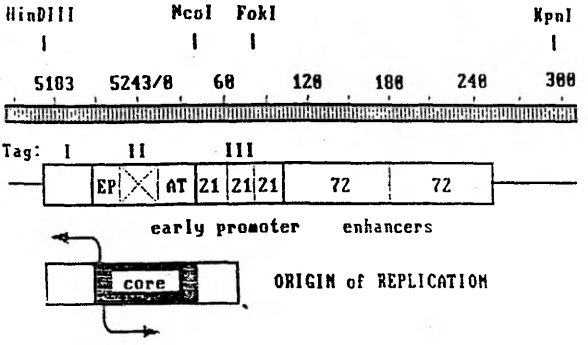
B

FIGURE 1 C



C

FIGURE 2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NcoI
FokI
KpnI
5183
5243/8
68
128
188
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308
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EP
aT
21
21
21
72
72
early promoter
enhancers
core
ORIGIN of REPLICATION

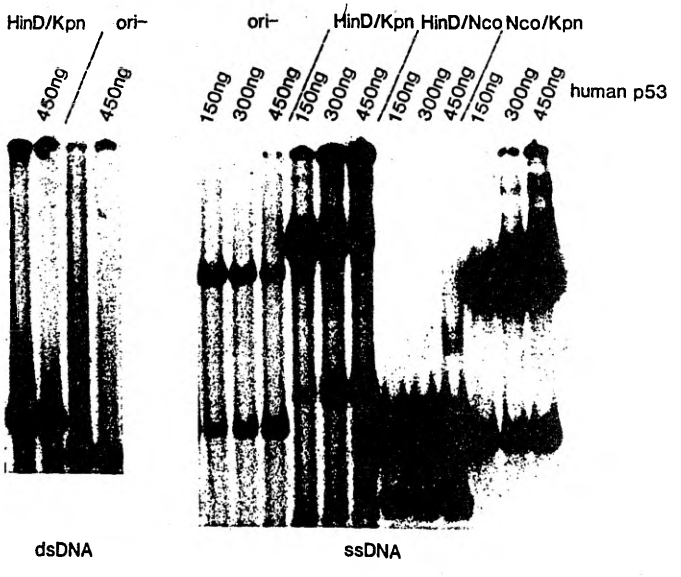

HinD/Kpn
ori-
ori-
HinD/Kpn
HinD/Nco
Nco/Kpn
450ng
450ng
150ng
300ng
450ng
150ng
300ng
450ng
150ng
300ng
450ng
150ng
300ng
450ng
human p53
dsDNA
ssDNA

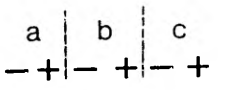
FIGURE 3

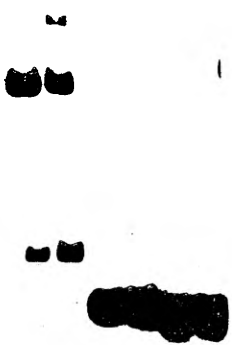
+: 200ng p53

a: Fok/Kpn
b: 72bp repeat(upper strand)
c: 72c: 72bp repeat(lower strand)

d: Fok/Kpn
e: Fok/Kpn-DdeI

FIGURE 4





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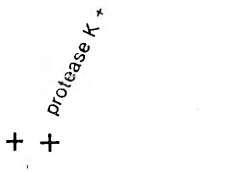

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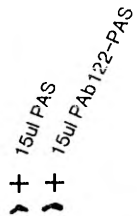


C

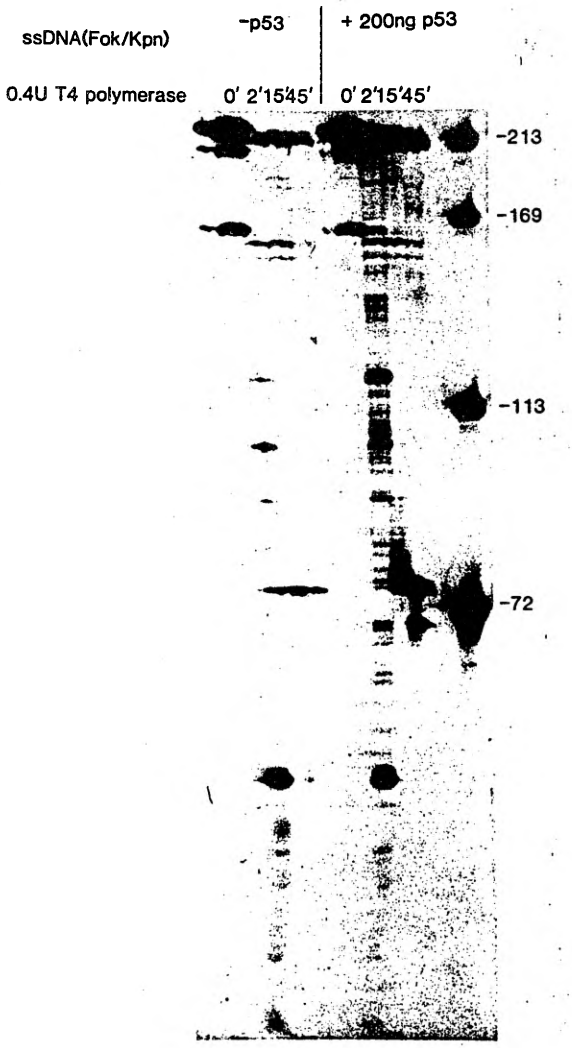

ssDNA(Fok/Kpn)
-p53
+ 200ng p53
0.4U T4 polymerase
0' 2'15'45'
0' 2'15'45'
-213
-169
-113
-72

FIGURE 5

Fok/Kpn(l) – 200ng p53 – 500 x excess A–J

A B C D E F G H I J

A: 140–154 / 212–226(u)
B: 276–290(l)
C: 107–178 / 179–250(u)
D: 107–178 / 179–250(l)
E: 165–173 / 237–245(l)
F: 161–167 / 233–239(l)
G: D, but G(173) A
H: D, but TGAAAGG(167–173) GCTCAGA
I: 75–146 / 179–217(u)
J: 75–146 / 179–217(l)

(u): upper strand
(l): lower strand

FIGURE 7

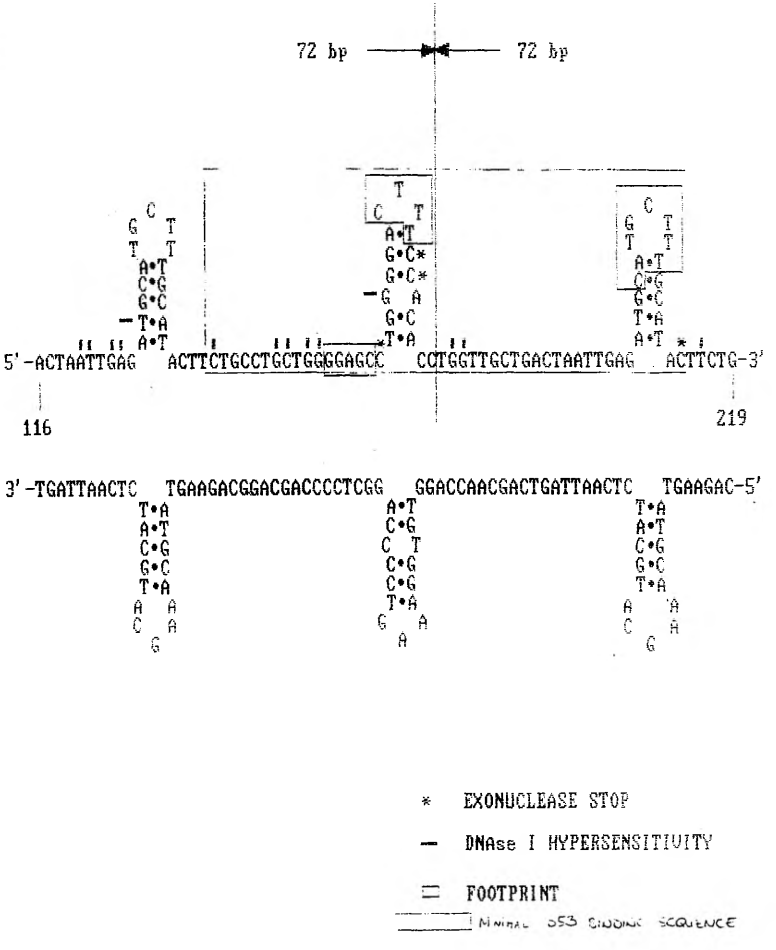
+: 200ng p53

a: Fok/Kpn (94-298)

b. 144-214

c: 164-214

FIGURE 8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72 bp
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116
219
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* EXONUCLEASE STOP
— DNAse I HYPERSENSITIVITY
= FOOTPRINT
MINIMAL SS3 BINDING SEQUENCE

Oncogene (1988), 3, 405–413

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Mouse p53 blocks SV40 DNA replication *in vitro* and downregulates T antigen DNA helicase activity

Horst-Werner Stürzbecher,[1] Ruth Brain,[1] Toivo Maimets,[2] Christine Addison,[1] Karran Rudge[1] & John R. Jenkins[1]

[1] *Cell Proliferation Labratory, Marie Curie Research Institute, The Chart, Oxted, Surrey, RH8 0TL, UK;* [2] *Estonian Biocenter, Taheiorn. Tartu, Estonia, 202400, USSR*

Immunopurified mouse p53 proteins were used to gain experimental access to the mechanisms underlying non-primate p53 directed supression of SV40 origin directed DNA replication *in vivo*. In replication competent HeLa cell extracts containing exogenous T antigen, mouse p53 blocks T antigen dependent DNA synthesis as *in vivo*. However, in transcription competent HeLa extracts, mouse p53 has no effect either on overall transcription or on the ability of immunopurified T antigen to down-regulate SV40 early transcription. We show that although mouse p53 has no significant effect on T antigen encoded activities such as ATPase and DNA binding, helicase activity is somewhat reduced suggesting that the *in vivo* suppression by mouse p53 of SV40 replication may be due, at least in part, to direct modulation of T antigen function.

Introduction

The cellular phosphoprotein p53, overproduced in a variety of neoplastic cell types (reviewed in Oren, 1985; Rotter & Wolf, 1985; Jenkins & Sturzbecher, 1988) was first identified by immunological techniques (Lane & Crawford, 1979; DeLeo *et al.*, 1979; Linzer & Levine, 1979). Evidence that p53 possesses intrinsic oncogenic potential comes from experiments which show that p53 expression constructs can immortalise both adult (Jenkins *et al.*, 1984) and embryo (Jenkins *et al.*, 1985; Rovinski & Benchimol, 1988) rodent cells, and can cooperate with an activated *ras* gene in the conversion of such cells to a fully transformed phenotype (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Finlay *et al.*, 1988; Parada *et al.*, 1984; Rovinski & Benchimol, 1988). Immortalization and *ras* complementation are distinct activities of p53 and can be separated by deletion mutagenesis (Jenkins *et al.*, 1985; Rovinski & Benchimol, 1988). The potential of wild-type p53 to score in transformation assays appears to be dependent upon powerful heterologous promoter/enhancers driving p53 expression (Jenkins *et al.*, 1985). However, p53 can be activated oncogenically by specific coding sequence mutations (Jenkins *et al.*, 1985) and activation seems to be associated with increased stability and overall steady state levels of the mutant proteins (Jenkins *et al.*, 1985; Stürzbecher *et al.*, 1987). p53 gene rearrangement, mutation, and deletion are frequent events in Friend leukemia virus induced malignancy (Rovinski *et al.*, 1987). p53 binds to SV40 T antigen and to the unrelated 57 Kdal protein product of the adenovirus E1b region (Lane & Crawford, 1979; Linzer & Levine, 1979); Sarnow *et al.*, 1982).

A number of mutant, but not wild-type, p53 proteins bind to cellular 70 Kdal heat-shock related proteins (hsp 72/73) (Hinds *et al.*, 1987; Stürzbecher *et al.* 1987; Jenkins *et al.*, 1988, *J. Virol.*, in press). Structural requirements for hsp 72/73 binding are clearly distinct from those implicated in T antigen binding and depend on p53 protein conformation (Stürzbecher *et al.*, 1988, *Mol. Cell. Biol.*, in press; Jenkins *et al.*, 1988, *J. Virol.* in press). T antigen-p53 complexes are found associated with both replicating and mature SV40 DNA in lytically infected cells (Tack *et al.*, 1986). In *in vitro* plate-binding assays, mouse p53 can both displace DNA polymerase α from complex with T antigen and exist as a trimeric T antigen-pol α-p53 complex (Gannon & Lane, 1987). Expression of mouse p53 in SV40 replication permissive monkey COS cells results in a profound inhibition of SV40-origin-dependent DNA replication (Braithwaite *et al.*, 1987). A variety of mouse p53 mutant proteins, competent to bind T antigen, also inhibit SV40 origin-directed DNA synthesis while hsp 72/73-binding p53 mutants do not. However, expression of wild-type human p53 in COS cells has no inhibitory effect whatsoever, indicating that suppression of replication reflects some intrinsic species difference between the way mouse and human p53 interact with T antigen (Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988b. We have analysed the suppression of SV40 DNA replication by mouse p53 in more detail and report here that T antigen-binding-competent mouse p53 inhibits SV40 DNA replication *in vitro* whereas hsp 72/73 binding p53 does not. Regulation of SV40 early and late transcription in *in vitro* assays is unaffected by p53. We show that origin specific DNA binding of T antigen to binding site II is slightly reduced by addition of immunoaffinity purified p53 whereas ATPase activity of T antigen remains undisturbed. Furthermore, mouse p53 suppresses T antigen helicase activity suggesting that inhibition of replication related biochemical activities of T antigen may be at least partially responsible for p53 mediated suppression of DNA replication.

Results

Mouse p53 inhibits SV40 DNA replication in vitro

We reported previously that T antigen binding mouse p53 acts as a suppressor of SV40 origin-directed DNA synthesis *in vivo* when introduced into SV40 permissive monkey COS cells, while hsp 72/73 binding mutant p53 fail to do so (Braithwaite *et al.*, 1987; Stürzbecher *et al.*, 1988b). To gain experimental access to the mechanisms

Correspondence: H.-W. Stürzbecher
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underlying inhibition of SV40 DNA replication by mouse p53, we reconstructed this activity in an *in vitro* DNA replication system as described by Li & Kelly (1984) using HeLa cell lysates and immunoaffinity purified T antigen and p53 protein. T antigen was isolated from transfected COS cells by circulating cell lysates through a column of PAb 419 anti T antigen antibody (Harlow *et al.*, 1981) covalently linked to protein A Sepharose and eluting T antigen at pH 10.8 after high salt washes as described by Simanis & Lane (1985). p53 purification from transfected COS cells was performed using an immunoaffinity column with covalently linked PAb 122 anti-p53 antibody. p53 was eluted from the antibody with excess peptide corresponding to the PAb 421/122 epitope as we described elsewhere (Wade-Evans & Jenkins, 1985). To separate the purified p53 from unbound peptide the eluate was passed through a Sephadex G25 column. Figure 1 shows different purified p53 proteins and T antigen. dl162p53 (Jenkins *et al.*, 1985) is an internal deletion mutant of mouse p53 which binds to T antigen and inhibits SV40 DNA replication *in vivo* (Braithwaite *et al.*, 1987; Stürzbecher *et al.*, 1988b); dl518p53 (Jenkins *et al.*, 1985) is a non-inhibiting hsp 72/73 binding mouse p53 mutant (Braithwaite *et al.*, 1987; Stürzbecher *et al.*, 1988b). Since transfected COS cells were the source for purifying p53, endogenous monkey p53 and associated T antigen are also present. However, because of overstaining, Figure 1 does not reflect the actual amounts of

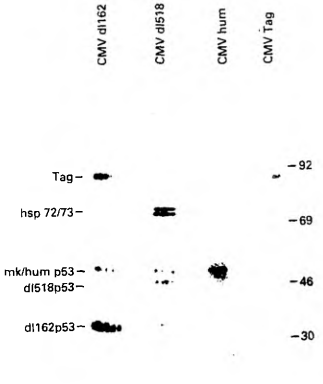


Figure 1 Immunoaffinity purified T antigen and p53 proteins. T antigen was purified from transfected COS cells as described by Simanis & Lane (1985). p53 proteins were purified from transfected COS cells by immunoaffinity chromatography using PAb 122 covalently linked to protein A Sepharose. p53 was eluted from the antibody with excess peptide corresponding to the PAb 122 epitope as described before (Wade-Evans & Jenkins, 1985). Tag, SV40 T antigen; hsp 72/73, heat-shock proteins 72/73 Kda; mk/hum p53, monkey/human p53; dl518p53, dl162p53, internal deletion mutants of mouse p53.

the different proteins. Western blot analysis revealed that the amount of mouse p53 in the preparations exceeds monkey p53 and T antigen about 50 fold (data not shown).

Purified dl162p53 and dl518p53 were tested for their effects on SV40 DNA replication *in vitro* (Figure 2A). SV40 form I DNA was incubated with a cytoplasmic HeLa cell lysate, 450 ng of purified T antigen and increasing amounts of p53 under conditions as described by Li and Kelly (Li & Kelly, 1984). After incubation for 90 min at 37°C in the presence of [α ^{32}P] dTTP the DNA was analysed by agarose gel electrophoresis. As a control for the specificity of the system, a plasmid carrying a defective SV40 origin of replication was analysed in the presence or absence of T antigen. Whereas SV40 DNA is readily replicated in the presence of T antigen, no replication occurs with the origin minus plasmid. Addition of 100 ng of dl162p53 protein to the system almost completely blocks SV40 DNA replication. On the other hand, introducing 100 ng of dl518p53 into the reaction has little effect on DNA replication. In addition, increasing amounts of p53 elution buffer do not inhibit the replication reaction. Equivalent experiments using immunopurified human p53 show that, as *in vivo*, human p53 does not inhibit replication (data not shown). From these results, we conclude that we have reconstituted the suppressor effect of mouse p53 on SV40 DNA replication *in vitro* and that this activity is restricted to T antigen binding competent p53 as it is *in vivo*.

The experiments described so far do not reveal at which stage during the reaction mouse p53 blocks SV40 DNA replication. No particular product of the *in vitro* replication reaction, neither supercoiled closed circular, nicked circular or linear SV40 DNA, nor replicating intermediates, accumulate after a 90-min replication reaction under dl162p53 block. We performed a time course based analysis of restriction fragments derived from [α ^{32}P] dTTP labelled replication intermediates (Figure 2B). Replication products were digested with excess BstNI restriction endonuclease which cuts 17 times in SV40 DNA (see Figure 2Ba) after 10, 20, 40, 60 and 90 min reaction times and approximately equal amounts of total cpm incorporated (6000–8000 cpm per time point) subjected to gel electrophoresis (Figure 2Bc). Incorporation of [α ^{32}P] dTTP into 300 ng of SV40 DNA was linear over the 90 min reaction period and was inhibited to about 50% in the presence of 60 ng dl162p53 (Figure 2Bb). Figure 2Bc shows that both in the presence and absence of dl162p53, restriction fragments corresponding to the SV40 origin of replication (Figure 2Bc; fragments G and I) are labelled already after 10 min reaction time whereas fragments near the termination (Figure 2Bc, fragments F, H) appear only after 40 min reaction time. These results indicate that the replication block imposed by mouse p53 must be localised either at or close to the initiation of DNA synthesis rather than during elongation or deconcatenation since there is no evidence for preferential labelling of restriction fragments at or near *ori*, or p53 must block several stages of replication to the same extent.

p53 does not affect transcription of SV40 DNA in vitro

An alternative explanation for the suppression of SV40 DNA replication by p53 *in vivo* (Braithwaite *et al.*,

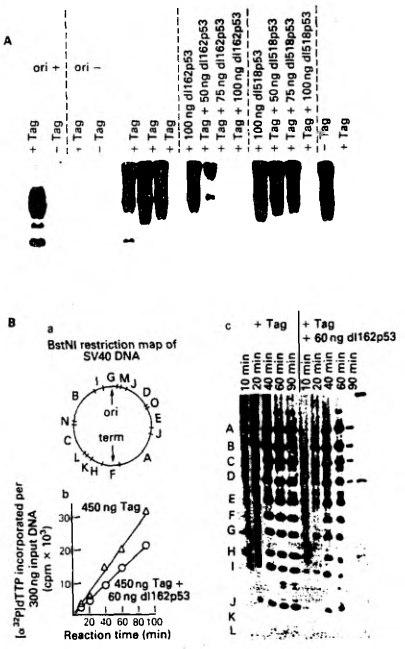
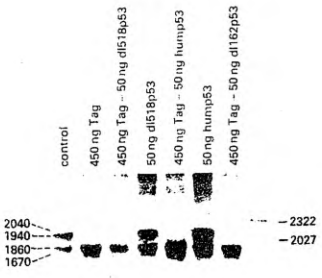


Figure 2 Inhibition of SV40 DNA replication by mouse p53 *in vitro*.
(A) The *in vitro* DNA replication reaction was performed as described by Li and Kelly (1984) using HeLa cell lysates, immunoaffinity purified T antigen and p53 protein (see Figure 1) and SV40 DNA.
ori⁺: SV40 DNA containing a function origin of replication.
ori⁻: SV40 DNA containing a defective origin of replication (Sfi I cut, blunt ended and religated).
Tag: +450 ng SV40 T antigen.
dl162d153: internal deletion mutant of mouse p53, competent to bind to T antigen.
dl518p53: internal deletion mutant of mouse p53, incompetent to bind to T antigen.
(B) (a) BstNI restriction map of SV40 DNA. Sixteen fragments ranging from 54 to 933 bp in length are shown. (b) Incorporation of [α ^{32}P] dTTP into SV40 DNA over the 90-min reaction period in absence and presence of 60 ng dl162p53. (c) Distribution of radioactivity in BstNI restriction fragments of SV40 DNA after labelling for various time periods. *In vitro* DNA synthesis in the presence of [α ^{32}P] dTTP was carried out for indicated time periods, the DNA subjected to cleavage by Bst NI, followed by polyacrylamide gel electrophoresis (6%, in Tris/borate/EDTA, pH 8.3) of restriction fragments. Amounts of radioactivity ranging from 6000 to 8000 cpm per lane was used

1987; Stürzbecher *et al.*, 1988b) is that p53 enhances transcription from plasmid DNA thus changing the equilibrium between transcriptionally active and replicating plasmid molecules, resulting indirectly in inhibition of plasmic replication. To address this question, we performed *in vitro* transcription assays as described by Handa *et al.*, (1981) using a HeLa cell lysate based eucaryotic transcription system. Figure 3 shows the result. Reactions containing HeLa lysate only produced [α ^{32}P]-UTP labelled early and late transcription from PstI cut SV40 DNA at a ratio of about 1:1 (Figure 3, control). Addition of 450 ng purified T antigen switched transcription almost completely from early to late products. Presence of 50 ng dl518p53 or human p53 (Figure 3) or up to 100 ng dl162p53 (not shown) had no influence on either amount or ratio of early and late transcripts. Introduction of T antigen plus either dl162p53, dl518p53 or human p53 into the system did not affect the T antigen dependent switch from early to late transcription (Figure 3). Titration analysis of T antigen and/or p53's produced similar results (data not shown). Transcription from the cytomegalovirus promoter (using SmaI cut CMVhump53-DNA), which drives p53 expression in constructs used for the *in vivo* studies of p53 suppression of SV40 origin-directed-DNA synthesis (Braithwaite *et al.*, 1987; Stürzbecher *et al.*, 1988b) was unaffected by either T antigen or p53 (data not shown).



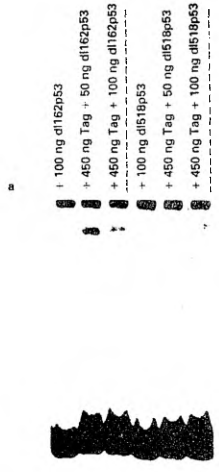


Figure 3 *In vitro* transcription of SV40 DNA is unaffected by p53. *In vitro* transcription reactions were performed in HeLa cell lysates using PstI cut SV40 DNA as template in presence or absence of T antigen (Tag) and/or purified dl162p53, dl518p53 and human p53. [α ^{32}P]-UTP labelled reaction products were analysed on 1.4% agarose gels in 10mM sodium phosphate (pH 6.8), 0.1% SDS.
2040, 1940: SV40 early transcripts
1860, 1670: SV40 late transcripts

Thus, we conclude that inhibition of SV40 DNA replication by p53 is not based on indirect effects of p53 on transcription regulation.

dl162p53 moderately reduces binding of T antigen to SV40 origin DNA binding site II

During lytic infection of SV40, T antigen is the only viral protein required for SV40 minichromosome replication (reviewed in De Pamphilis & Bradley, 1986). Specific binding of T antigen to the SV40 origin of replication is absolutely required to start the synthesis of each daughter DNA molecule (Deb *et al.*, 1986; Margolskee & Nathans, 1984; Paucha *et al.*, 1986; Cole *et al.*, 1986; Li *et al.*, 1986). A trivial explanation for the inhibition of SV40 DNA replication by mouse p53 might therefore be that p53 is sequestering T antigen from its binding sites at the origin of replication thus preventing initiation of DNA synthesis. To test this hypothesis, gel retardation assays were performed using end labelled HindIII to KpnI fragment of SV40 DNA containing all three T antigen binding sites on SV40 (DeLucia *et al.*, 1983; Tegtmeyer *et al.*, 1983). To reach binding equilibrium the reaction was carried out for 1 h at 37°C under replication conditions as described by Deb & Tegtmeyer (1987). Figure 4a shows the results. Binding of T antigen to the complete origin fragment was basically unaffected by increasing amounts of T antigen binding dl162p53 compared to hsp 72/73

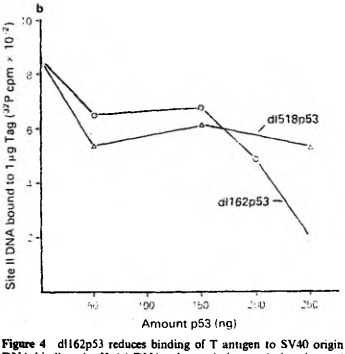


Figure 4 dl162p53 reduces binding of T antigen to SV40 origin DNA binding site II. **(a)** DNA gel retardation analysis using end labelled HindIII to KpnI SV40 DNA fragment (nucleotides 5171 to 294) containing all three T antigen binding sites at the origin of DNA replication. Binding reactions were performed under *in vitro* replication conditions for 1 h at 37°C (for details see Materials and methods) and the reaction mixtures analysed directly on a neutral 8% polyacrylamide gel. **(b)** Nitrocellulose filter binding assay using an end labelled oligonucleotide corresponding to T antigen binding site II representing the minimal origin of replication. DNA binding was performed for 30 min at 37°C under DNA helicase conditions in absence of ATP with increasing amounts of dl162p53 or dl518p53 present. Reaction mixtures were applied to nitrocellulose filters, the filters washed and analysed for retained [^{32}P]-labelled DNA by liquid scintillation counting (for details see Materials and methods)

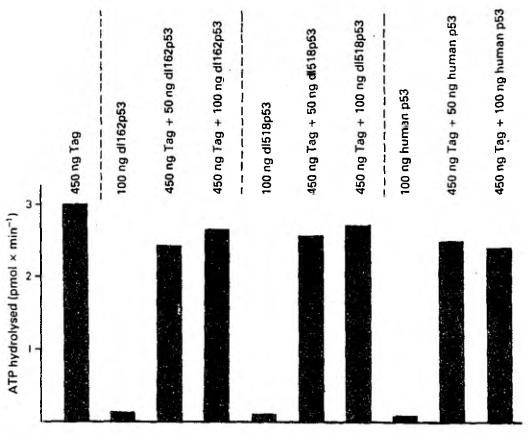


Figure 5 Immunopurified p53 does not affect T antigen ATPase activity. T antigen ATPase assays in absence or presence of dl162p53, dl518p53 or wild-type human p53 were performed for 10 min at 20°C in presence 0.5 μCi [γ-^{32}P] ATP. Unreacted ATP was precipitated with acid washed charcoal and the supernant analysed for free [$^{32}P_i$] by Cerenkov counting

binding dl518p53 (Figure 4a). However, only T antigen binding region II DNA is crucial to origin function in the initiation of DNA replication (Deb *et al.*, 1986b). Since region I is the strongest T antigen binding site (De Pamphilis & Bradley, 1986) inhibition of T antigen binding to site II by p53 could have been masked in the DNA retardation assay. Therefore nitrocellulose filter binding assays were performed using a [^{32}P] end labelled 65 bp DNA fragment corresponding to binding site II with additional 20 bp flanking polylinker sequences. The results are shown in Figure 4b. Addition of up to 250 ng dl518p53 does not perturb T antigen binding to site II. On the other hand, presence of more than 200 ng dl162p53 is required to cause a moderate reduction in site II binding with about 50% inhibition by 250 ng dl162p53. Thus, although binding site II is identical to the minimal origin of SV40 DNA replication and T antigen binding to this site is essential for initiation of DNA synthesis the observed reduction of T antigen binding caused by dl162p53 is not sufficient to explain its suppression of SV40 DNA replication.

T antigen ATPase activity is unaffected by immunopurified p53

In addition to its initiation function, T antigen also plays a role in the elongation process of viral DNA synthesis, particularly in the process of parental strand separation via its intrinsic ATPase activity (Stahl *et al.*, 1985; Wiekowski *et al.*, 1987; Giacherio & Hager, 1979; Clark *et al.*, 1981). In a previous report, Tack *et al.* (1988) showed that p53-bound T antigen from SV40 infected monkey CV1 cells on immunocomplexes with antibody PAb 122 exhibits a 2- to 5-fold increased ATPase activity compared to free T antigen. However, the authors could not exclude steric interference of the antibodies with T antigen ATPase activity. Therefore we tested whether immunoaffinity purified p53 affects the T antigen intrinsic ATPase activity. The results of T antigen ATPase assays, performed as described by Clark *et al.* (1981) using immunoaffinity purified T antigen and dl162p53, dl518p53 or wild-type human p53 are shown in Figure 5. Neither immunopurified mouse nor human p53 modulates T antigen ATPase activity in our hands. The very low ATPase activity in the p53 preparations are due to small amounts of T antigen from COS cells. Thus, immunoaffinity purified mouse and human p53 does not modulate the ATPase activity of T antigen, indicating that inhibition of *in vitro* SV40 DNA replication by dl162p53 is not caused by perturbations of this T antigen activity.

dl162p53 inhibits T antigen helicase activity

During SV40 DNA replication, T antigen also catalyses an ATP-dependent unwinding of circular SV40 ori$^+$ duplex DNA, allowing the host cell DNA replication machinery access to the DNA, and subsequently acts as a helicase to wind the DNA helix in advance of the replication fork (Stahl *et al.*, 1986; Dean *et al.*, 1987; Goetz *et al.*, 1988; Wiekowski *et al.*, 1988). In the following experiment we determined T antigen helicase activity in the absence or presence of purified dl162p53 or dl518p53. As a substrate for DNA helicase, we used single stranded M13mp18 DNA, carrying an annealed [^{32}P]-labelled 19-mer oligonucleotide. 750 ng purified T antigen was incubated for 60 min with this substrate at 37°C under standard conditions for T antigen helicase assays (Stahl *et al.*, 1986) in the presence of increasing amounts of purified mouse p53. The result is shown in Figure 6. With increasing amounts of dl162p53 present in the reaction T antigen unwinding activity was blocked to 65%, whereas dl518p53 did not affect the T

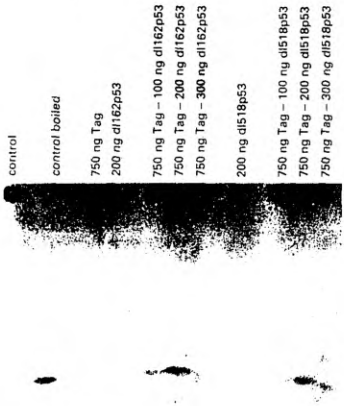


Figure 6 dl162p53 inhibits T antigen helicase activity. As a DNA substrate, an [α ^{32}P] dATP labelled oligonucleotide-primed M13mp18 DNA was used in a T antigen helicase reaction with constant amounts of T antigen and increasing amounts of either dl162p53 or dl518p53. After 60 min reaction at 37°C products were analysed by polyacrylamide gel electrophoresis (6% acrylamide) and autoradiography

antigen catalysed release of hydrogen-bonded 19-mer oligonucleotide from M13mp18 DNA. Neither dl162p53 and dl518p53 exhibited any helicase activity by themselves. From these findings we conclude that suppression of SV40 DNA replication by mouse p53 is at least partially caused by interference with T antigen helicase activity.

Discussion

In previous publications (Braithwaite *et al.*, 1987; Stürzbecher *et al.*, 1988b) we have shown that mouse p53, when transiently expressed in SV40 transformed monkey COS cells, profoundly inhibits SV40 origin directed DNA synthesis *in vivo*. This suppressor activity is restricted to non-primate p53 T antigen binding since neither non T antigen but hsp 72/73 binding mouse p53, nor human 53 cause this effect. We demonstrate here that immunoaffinity purified T antigen-binding mouse p53 blocks SV40 DNA replication *in vitro* but has no effect on the regulation *in vitro* of SV40 early and late transcription by T antigen.

T antigen plays a dual role in SV40 DNA replication. It initiates each round of replicative DNA synthesis by binding to the origin of replication followed by opening the DNA helix at the origin, thus allowing the cellular DNA replication machinery access to the DNA. Secondly, during the elongation step of DNA replication, it acts as a helicase to unwind the DNA helix by moving along the template for leading strand synthesis in concert with DNA polymerase α, combining fork movement with the process of DNA synthesis. T antigen intrinsic biochemical properties required to fulfill these biological functions are SV40 origin-specific DNA binding (Tjian, 1978; DeLucia *et al.*, 1983; Tegtmeyer *al.*, 1983); ATPase (Clark *et al.*, 1981; Giacherio Hager, 1979) and DNA helicase activities (Stahl *et a* 1986; Dean *et al.*, 1987). In addition, an interactic between T antigen and DNA polymerase α has bee detected by immunological procedures (Smale & Tjia 1986). Inhibition of SV40 DNA synthesis by mouse p5 *in vivo* and *in vitro* correlates very well with the abilit of p53 to bind to T antigen (Braithwaite *et al.*, 198 Stürzbecher *et al.*, 1988b, this publication), indicatin that p53 might interfere with the replication process b disturbing normal T antigen functions. *In vitro* repl cation experiments (Figure 2A) and time course base analysis of replication products (Figure 2B) show tha no particular form of replication product accumulate due to p53 interference. This suggests that p53 eithe blocks a very early step in the initiation process o affects several stages of DNA replication. The result shown here are consistent with the latter idea. While th ATPase activity of T antigen (Figure 5) and regulatio of SV40 early and late transcripts by T antigen (Figur 3) appear to be unaffected, p53 does moderately reduc binding of T antigen to the origin of replication (Figur 4) and it causes inhibition of T antigen helicase activity (Figure 6). In addition, mouse p53 is capable of displacing DNA polymerase α from T antigen-pol α complexes *in vitro* (Gannon & Lane, 1987). Whether these findings are sufficient to explain the underlying mechanisms of suppression of SV40 DNA replication by mouse p53 *in vivo* and *in vitro* is not entirely clear for methodological reasons. For example, 100 ng of dl162p53 blocks *in vitro* SV40 DNA replication performed with 450 ng of T antigen almost completely. However, the amount of p53 necessary for efficient inhibition of T antigen helicase activity is three to five fold higher.

Although the results presented here provide evidence for biochemical effects of p53 on T antigen mediated SV40 DNA replication, the main concern remains the biological relevance of a heterologous system in which p53 from a species non-permissive for SV40 DNA replication interferes with DNA synthesis in either permissive cells or in semipermissive cell lysates. A simple explanation for these findings could be that mouse p53 blocks normal T antigen functions and/or occupies binding sites for other cellular factors like DNA polymerase α because it complexes more tightly with T antigen than human and monkey p53 do (Harlow *et al.*, 1981). The p53 binding site on T antigen (amino acid residues 272 to 517; Schmieg & Simmons, 1988) overlaps with the sequences necessary for T antigen to bind to DNA polymerase α *in vitro* (approximate residues 335 to 626: Smale & Tjian, 1986; Gannon & Lane, 1987) and to bind to ATP (residues 418 to 528; Bradley *et al.*, 1987; Smale & Tjian, 1986), but it maps outside of the core region of the origin-specific DNA-binding domain (residues 140 to 281; Paucha *et al.*, 1986; Simmons, 1986) and overlaps only slightly with the origin binding domain including the zinc-finger-motif of T antigen (residues 140 to 371; Simmons, 1988). Blocking experiments using a panel of monoclonal anti T antigen antibodies have revealed that the ATPase domain, the DNA binding domain and also region at the N-terminus are required for T antigen helicase activity (Wiekowski *et al.*, 1987). Judged from these data the interpretation that tight binding of mouse p53 to T

antigen interferes with normal T antigen function seems justified. On the other hand, our laboratory has created point mutants of mouse p53 which do not inhibit SV40 DNA replication *in vivo* yet bind T antigen as tightly as wild-type mouse p53 (Rudge *et al.*, in preparation). These data imply that T antigen binding is necessary but insufficient for p53 to act as a suppressor of SV40 DNA synthesis. These observations may be the first indication of an active role for p53 in regulation of DNA replication and, together with the fact that expression of wild-type non-primate but not human, p53 proteins can inhibit SV40 replication, offers an explanation of the apparent contradiction that p53 can be mutationally activated scores as a dominant transforming gene in transfection assays (Jenkins *et al.*, 1984, 1985; Eliyahu *et al.*, 1984; Parada *et al.*, 1984; Finlay *et al.*, 1988; Rovinski & Benchimol, 1988) yet is functionally absent in several transformed cell lines (Wolf *et al.*, 1984; Wolf & Rotter, 1985) and the p53 gene selectively lost during Friend virus mediated leukemogenesis (Rovinski *et al.*, 1987). Any functionally defective p53 proteins which retain the ability to complex with some cellular target could compete with wild-type p53 for these sites and it may be significant that all the activated p53 mutants that we have analysed are stable and overexpressed relative to wild-type p53 (Jenkins *et al.*, 1985; Stürzbecher *et al.*, 1988). In that context, wild-type mouse p53 when introduced into monkey COS cells could be considered a transdominant activated 'mutant p53' in this heterologous system which inhibits SV40 DNA replication via interference with T antigen DNA binding, helicase activity and displacement of DNA pol α from T antigen. A cellular helicase has been detected in complex with DNA polymerase α holoenzyme (Hübscher & Staider, 1985), perhaps indicating an analogous mechanism at forks of replicating chromosomal DNA. p53 as an oncogene may therefore act by perturbing either positively or negatively the recruitment of a subset of cellular DNA replication origins or the processivity of ongoing DNA synthesis.

Materials and methods

Protein purification

T. antigen. For T antigen purification SV40 transformed monkey COS cells (Gluzman, 1981) were transfected with a T antigen expression vector, constructed in the pBC12CMV vector described by Cullen (1986), using the DEAE-dextran transfection protocol as described previously (Stürzbecher *et al.*, 1987). The cells were harvested 82 h after transfection and used for T antigen preparation by the immunoaffinity procedure of Simanis & Lane (1985) resulting in essentially pure T antigen as shown in Figure 1.

p53. For p53 purification COS cells were transfected with either CMVdl162, CMVdl518 or CMVhump53 (Jenkins *et al.*, 1985; Stürzbecher *et al.*, 1987; Braithwaite *et al.*, 1987). CMVdl162 encodes for T antigen binding competent mouse dl162p53, CMVdl518 for lisp 72/73 binding mouse dl518p53 and CMVhump53 for wild-type human p53 (Harlow *et al.*, 1985). 82 h post transfection, p53 was immunoaffinity purified using PAb 122 monoclonal anti p53 antibody (Guerney *et al.*, 1980), covalently linked to protein A Sepharose as described previously (Wade-Evans & Jenkins, 1985). Purified p53 was eluted from the antibody with excess peptide corresponding to the epitope for PAb 112 (Lys-Lys-Gly-Glu-Ser-Thr-Ser-Arg-His-Lys). Subsequently, p53 and excess peptide were separated over a Sephadex G25 column.

In vitro DNA replication. In vitro SV40 DNA replication assays were performed as described by Li and Kelly (Li & Kelly, 1984). Reaction mixtures (50 μl) contained (final concentrations) 40 mM Hepes-KOH (pH 7.5), 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μM each dGTP, dCTP and dATP, 25 μM [α ^{32}P] dTTP (~2000 cpm/pmol), 3 mM ATP, 200 μM each CTP, UTP and GTP, 40 mM creatine phosphate, and 1 μg creatine phosphokinase. Standard reaction mixtures also contained 0.3 μg of SV40 form I DNA, 0.45 μg of T antigen and 150 to 200 μg of HeLa cell cytoplasmic extract prepared as described by Stillman & Gluzman (1985). Reaction mixtures were prepared on ice and subsequently incubated at 37°C for 90 min. Reactions were terminated by addition of 10 mM EDTA and 0.1% sodium dodecyl sulphate. Mixtures were incubated for 30 min at 37°C in the presence of proteinase K (final concentration 1 mg ml^{-1}) and then extracted twice with phenol/chloroform. The DNA was separated from unincorporated nucleoside triphosphates by spin dialysis with Sephadex G-50. The DNA was then ethanol precipitated and analysed by agarose gel electrophoresis. For restriction fragment analysis, replication products were digested with excess BstNI restriction endonuclease and the digestion products subjected to polyacrylamide gel electrophoresis.

In vitro transcription. Transcription assay was done using commercial Eucaryotic Transcription System (BRL) by the method of Handa *et al.* (1981). Reactions were performed in 50 μl containing 30 μl HeLa cell lysate, 0.14 mM EDTA, 1 mM creatine phosphate, 0.5 mM of each rATP, rCTP, rGTP, 0.5 mM UTP and 15 μCi [α-^{32}P] UTP. Purified dl162, dl518, human p53 and/or T-antigen was added and reaction was started by adding 2.5 μg DNA template (either PstI cut SV40 or SmaI cut CMVhump53). After incubation at 30°C for 1 h the reactions were stopped with 250 μl 8 M urea, 0.5% SDS, 10 mM EDTA and treated with 300 μl phenol-chloroform (1:1). Phenol phase was re-extracted with 150 μl 7 M urea, 0.35 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS and 25 μg tRNA and free nucleotides eliminated by three ethanol precipitations. Final RNA pellet was taken up in 40 μl glyoxylation mix (50% DMSO, 1 M glyoxal, 10 mM sodium phosphate pH 6.8), incubated 1 h at 50°C and analysed on 1.4% agarose gel in 10 mM sodium phosphate (pH 6.8), 0.1% SDS.

SV40 origin DNA-binding. DNA-binding was performed under *in vitro* replication conditions as described by Deb & Tegtmeyer (1987). Standard mixtures (30 μl) contained 30 mM Hepes pH 7.5, 1 mM DTT, 0.1 mg of bovine serum albumin per ml, 8% glycerol, 7 mM $MgCl_2$, 40 mM creatine phosphate, 4 mM ATP and 2 μg of creatine phosphokinase. Reaction mixtures also contained end labelled SV40 origin DNA sequences extending from the HindIII site at nucleotide position 5171 to the KpnI site at nucleotide 294 and 450 ng of T antigen. Binding reactions were carried out for 1 h at 37°C. For DNA gel retardation assays the reaction mixtures were analysed directly on a neutral 5% polyacrylamide gel.

Nitrocellulose filter DNA binding assays. Nitrocellulose filters (2.5 cm diameter, 0.45 μm pore size) were washed in 0.3 M NaOH for 30 min and rinsed three times with 0.1 M Tris/HCl pH 7.6. Immediately before use, filters were washed with 0.5 ml of ice-cold washing buffer (20 mM $NaPO_3$ pH 7.3, 3% β-mercaptoethanol, 0.1 mM EDTA). Reaction mixtures, final vol 50 μl, contained 20 mM Tris/HCl, pH 7.6, 25 mM NaCl, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, [^{32}P]-labelled binding site II DNA and 10 ng of competitor DNA. After mixing, 1 μg of T antigen and indicated amounts of dl162p53 or dl518p53 were added. The reaction mixtures were incubated at 37°C for 30 min and

applied to the nitrocellulose filters. The filters were washed twice with 0.5 ml of ice cold washing buffer, dried, and analysed for retained [^{32}P]-labelled DNA by Cerenkov radiation counting.

ATPase assays. ATPase assays were performed as described by Clark *et al.* (1981) in 20 μl of buffer containing 25 mM PIPES (1,4 piperazinediethanesulphonic acid), pH 7.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Nonidet-P40, 5 μM unlabelled ATP, and 0.5 μCi of [γ ^{32}P] ATP. Reactions were carried out with 100 ng of T antigen for 10 min at 20°C. Unreacted ATP was precipitated with 100 μl of acid-washed charcoal (7.5% W/U in 50 mM HCl, 5 mM H_3PO_4). The charcoal was pelleted by centrifugation and 90 μl of supernatant was analysed for free [$^{32}P_i$] by Cerenkov radiation counting.

DNA helicase assays. Substrates: 50 ng of a 15-mer universal primer (3'-ATGTTGCAGCACTGA-5') was annealed to 250 ng of M13mp18 DNA under the conditions used for DNA sequencing (Sanger *et al.*, 1980). Subsequently, the primer was elongated in the presence of [α ^{32}P] dATP using the DNA polymerase I Klenow fragment as described by Stahl *et al.* (1986). The 40-μl standard reaction mixtures contained 20 mM Tris/HCl pH 7.5; 7.5 mM $MgCl_2$; 1 mM dithiothreitol; 2 mM ATP; 1 mg/ml phosphocreatine; 0.1 mg/ml creatine phosphokinase and 6–9 ng of labelled substrate. 750 ng T antigen (and various amounts of dl162p53 or dl518p53) were added and the reaction mixture incubated for 60 min at 37°C. The reaction was stopped by addition of 0.1 volume of 3% sodium dodecylsulfate and 0.5 M EDTA and 50% glycerol. Reaction products were analysed by polyacrylamide electrophoresis (6%) in Tris/borate EDTA buffer, pH 8.3. Helicase activity was quantitated by measuring the radioactivity of the DNA fragments still annealed after reaction by scintillation counting.

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Identification and Analysis of Human p53 Mutants That Are *trans*-Dominant Modulators of DNA Replication In Vivo

J.R. Jenkins,* H.-W. Stürzbecher,* R. Brain,* M. Grimaldi,* T. Maimets,† K. Rudge,* W. Court,* and C. Addison*

*Cell Proliferation Laboratory, Marie Curie Cancer Research, Oxted, Surrey, RH8 0TL, United Kingdom; †Estonian Biocenter, Tähetorn, Tartu, Estonia 202400, Union of Soviet Socialist Republics

We investigated the ability of mouse and human mutant p53 proteins to perturb replication of the SV40 chromosome in vitro and in transiently transfected COS cells. Mouse p53 inhibited SV40 DNA replication both in vivo and in vitro, but inhibition was abolished by a single point mutation having no detectable effect on T-antigen-p53 complexing. Human wild-type p53 failed to inhibit SV40 DNA replication in vivo or in vitro. However, two point mutants involving phosphoserine residues both inhibited SV40 replication in vivo, whereas a linker insertion mutant stimulated replication. We discuss these results in relation to SV40 DNA replication and their implications for a possible mechanism by which abnormalities of p53 expression might contribute to a loss of growth control.

p53 is a short-half-life, low-abundance nuclear phosphoprotein that is present at elevated levels in cells transformed by a wide variety of insults including viruses, chemicals, and radiation (for reviews, see Crawford 1983; Oren 1985; Rotter and Wolf 1985; Jenkins and Stürzbecher 1988). Murine p53 expression constructs can immortalize both adult (Jenkins et al. 1985) and primary embryo (Rovinski and Benchimol 1988) rodent cell cultures and can cooperate with an activated Ha-*ras* gene in the conversion of such cells to a malignant phenotype (Eliyahu et al. 1984; Jenkins et al. 1984; Parada et al. 1984). Coding sequence mutations can enhance the oncogenic potential of p53 (i.e., p53 can be mutationally activated) (Jenkins et al. 1985), and such enhancement is frequently associated with a significant increase in the half-life of the encoded p53 protein (Jenkins et al. 1985; Stürzbecher et al. 1988a). Immortalization and *ras* complementation are distinct activities of p53 and can be separated by deletion mutagenesis (Jenkins et al. 1985; Rovinski and Benchimol 1988). Immunologically abnormal p53 proteins are present in some human tumor-derived cell lines (Maimets and Jenkins 1987). Both alleles of the p53 gene are deleted in the human myeloid tumor-derived cell line HL-60 (Wolf and Rotter 1985), and p53 gene rearrangements have been detected in some human osteogenic sarcomas (Masuda et al. 1987). p53 gene rearrangement and deletion are common features of cell lines derived from Friend-leukemia-virus-transformed cells (Rovinski et al. 1987; Ben David et al. 1988). Some mutant, but not wild-type, p53 proteins complex with the heat-shock-related proteins hsp72/73 (Hinds et al. 1987; Stürzbecher et al. 1987, 1988a). However, hsp72/73 binding is not reliably diagnostic of either mutation or oncogenic activation (Stürzbecher et al. 1988a). Relative overexpression of p53 protein is detectable in some human primary breast cancers, and, possibly uniquely among the known oncogenes, a subset of human cancer patients have high levels of circulating autoantibodies against p53 (Crawford et al. 1982; Caron de Fromentel et al. 1987). p53 forms specific complexes with the SV40 T antigen (Lane and Crawford 1979), and it is probable that, when in complex with p53, T antigen is usurping the role of some cellular "T antigen equivalent" protein, since the protein domains comprising the T-antigen-binding site on p53 are both structurally and functionally conserved between human, mouse, and *Xenopus* sp. (Jenkins et al. 1988). With the exception of T antigen, SV40 relies exclusively on cellular factors for viral DNA replication, and in a previous report, we showed that mouse p53 could disrupt SV40 DNA replication in vivo when this protein was expressed in SV40 replication-permissive monkey cells (Braithwaite et al. 1987; Stürzbecher et al. 1988c). The 30-fold decrease in viral DNA replication that we observed suggested a possible involvement of p53 in viral DNA synthesis and indicated that SV40 DNA replication might serve as a model system from which we could gain insights into the normal cellular function(s) of p53 and how aberrations of p53 expression might contribute to the loss of growth control characteristic of malignancy. We are therefore undertaking a detailed investigation of the properties of wild-type and mutant p53 proteins, with special regard to human p53, in SV40 replication systems both in vivo and in vitro. We report here on the identification and preliminary characterization of two novel classes of human p53 mutant, one of which suppresses, the other of which stimulates, SV40 origin-dependent DNA replication in vivo. By analogy with these effects, our data suggest that mutant p53 proteins or loss of p53 expression may exert oncogenic activity by perturbing either positively or negatively the onset or progression of replicative DNA synthesis of a subset of mammalian cell replicons.

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Methods

Cell culture and transfection

Monkey COS cells (Gluzman 1981) were used as the recipient cell line in all transfection experiments. Details for DEAE/Dextran transfection are as given by Stürzbecher et al. (1987).

Plasmids

All human (Harlow et al. 1985) and mouse (Jenkins et al. 1984) wild-type and mutant p53 cDNAs were cloned into the pBC12CMV vector described by Cullen (1986). To create a library of point mutants of mouse p53 cDNA, the coding strand of wild-type mouse p53 in ssM13 DNA was treated with methoxylamine, resulting in 7-methoxy-dC. After annealing to unmutagenized noncoding strand, the double-stranded p53 fragment was transferred into the pBC12CMV vector, and the plasmids were transfected into competent *Escherichia coli* DH5 (Hanahan 1983). During plasmid replication in *E. coli*, 7-methoxy-dC generates C → T transitions in the coding strand of p53 cDNA. Mouse p53 mutants *dl* 162 and *dl* 518 have been described previously (Jenkins et al. 1985). CMVhup315$_{ala}$ contains GCT at codon 315, changing the serine residue in wild-type human p53 to alanine. CMVhup392$_{ile}$ contains ATC at codon 392, resulting in a serine-to-isoleucine transition. CMVhuins615 contains a *Bgl*II 12-mer linker insertion (New England Biolabs) at the corresponding site.

Monoclonal antibodies

The hybridomas PAb 421, PAb 419, PAb 423 (Harlow et al. 1981), PAb 200-47 (Dippold et al. 1981), PAb 242, PAb 246, and PAb 248 (Yewdell et al. 1986) were generously provided by D.P. Lane (Imperial Cancer Research Fund). The monoclonal N33F3-5 was a gift from W.J. Welch.

Replication analysis of plasmid DNA

Plasmid DNA was isolated 72 hours posttransfection, using modified Hirt supernatant procedures (Hirt 1967). Plasmid DNA was digested with *Dpn*I restriction endonuclease, and digests were used to transform competent DH5 *E. coli* (Hanahan 1983). *Dpn*I-resistant DNA molecules representing the replicated fraction (Li and Kelly 1985) transform *E. coli* efficiently and are thus a measure of plasmid replication. Cells were plated on L-agar supplemented with 0.01 M $MgCl_2$ and 50 μg ml^{-1} ampicillin and incubated overnight at 37°C. Colonies were counted manually

Extraction of cells and sucrose gradient analysis

Radiolabeling, lysis of transfected COS cells, immunoprecipitation, and analysis by SDS-PAGE were carried out as described previously (Stürzbecher et al. 1987). For sucrose gradient analysis, 0.9 ml of labeled cell extracts were layered on top of 10 ml of linear 5–20% sucrose gradient (10 mM Tris-HCl [pH 7.3], 0.1 M NaCl, 0.5% NP-40) on a cushion of 0.4 ml of 60% sucrose. Centrifugation was performed in a Sorvall TST-41 rotor at 36,000 rpm for 14 hours. Thyroglobulin (18S), catalase (10S), and IgG (7S) were run in parallel gradients as markers for sedimentation coefficients.

In vitro association between p53 and T antigen

p53 cDNA genes under the control of the T7 promoter were transcribed in vitro with T7 polymerase. Reaction mixtures containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT), and 0.5 mM ribonucleotides were incubated for 1 hour at 40°C. Translation of cRNA products was carried out in the mRNA-dependent rabbit reticulocyte lysate system by using [^{35}S]methionine as the label (Wade-Evans and Jenkins 1985). After 60 minutes reaction time at 32°C, translation mixtures were incubated with 1.5 μg of immunopurified T antigen (Simanis and Lane 1985) in 1 ml of RIPA buffer minus SDS for 1 hour at room temperature.

Protein purification

For T-antigen purification, COS cells (Gluzman 1981) were transfected with a T-antigen expression plasmid, constructed in the pBC12CMV vector (Cullen 1986). The cells were harvested 82 hours posttransfection and used for T-antigen preparation by the immunoaffinity procedure of Simanis and Lane (1985), resulting in essentially pure T antigen.

For p53 purification, COS cells were transfected with either CMVdl162 or CMVdl518 (Braithwaite et al. 1987). p53 was immunoaffinity purified 82 hours posttransfection using PAb 122 monoclonal anti-p53 antibody (Gurney et al. 1980) covalently linked to protein-A-Sepharose as described elsewhere (Wade-Evans and Jenkins 1985; Stürzbecher et al. 1988b). p53 was eluted from the antibody with excess peptide corresponding to the epitope for PAb 122.

In vitro SV40 DNA replication

In vitro SV40 DNA replication assays were performed as described previously by Li and Kelly (1984). Reaction mixtures (50 μl) contained (final concentrations) 40 mM HEPES-KOH (pH 7.5); 8 mM $MgCl_2$; 0.5 mM DTT; 100 μM each dGTP, dCTP, and dATP; 25 μM [α-^{32}P]dTTP (~2000 cpm/pmol); 3 mM ATP; 200 μM each CTP, UTP, and GTP; 40 mM creatine phosphate; and 1 μg of creatine phosphokinase. Standard reaction mixtures also contained 0.3 μg of SV40 form I DNA, 0.45 μg of T antigen, and 150–200 μg of HeLa cell cytoplasmic extract prepared as described previously by Stillman and Gluzman (1985).

Nitrocellulose filter DNA-binding assay

Nitrocellulose filters were washed in 0.3 M NaOH for 30 minutes and rinsed three times with 0.1 M Tris-HCl (pH 7.6). Immediately before use, filters were washed with 0.5 ml of ice-cold washing buffer (20 mM $NaPO_3$ [pH 7.3], 3% β-mercaptoethanol, and 0.1 mM EDTA). Reaction mixtures contained 20 mM Tris-HCl (pH 7.6), 25 mM NaCl, 7.5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml bovine serum albumin, ^{32}P-labeled binding site II DNA, and 10

ng of competitor DNA. After mixing, 1 μg of T antigen and indicated amounts of *dl*162p53 or *dl*518p53 were added. The reaction mixtures were incubated at 37°C for 30 minutes and applied to the nitrocellulose filters. The filters were washed twice with 0.5 ml of washing buffer, dried, and analyzed for retained ^{32}P-labeled DNA.

DNA helicase assay

Under the conditions used for DNA sequencing, 50 ng of a 15-mer universal primer (3'-ATGTTGCAG-CACTGA-5') was annealed to 250 ng of M13mp18 DNA (Sanger et al. 1980). The primer was elongated in the presence of [α-^{32}P]dATP using the DNA polymerase I Klenow fragment as described previously by Stahl et al. (1986). Standard reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 1 mg/ml phosphocreatine, 0.1 mg/ml creatine phosphokinase, and 6–9 ng of labeled substrate. T antigen (750 ng) (and various amounts of *dl*162p53 or *dl*518p53) was added, and the reaction mixture was incubated for 60 minutes at 37°C. Reaction products were analyzed by polyacrylamide electrophoresis (6%) in Tris/borate/EDTA buffer (pH 8.3).

Results

Effects of mouse p53 on SV40 DNA replication in vitro

To gain experimental access to the mechanisms underlying the suppression of SV40 DNA replication by mouse p53, we reconstructed this activity in the in vitro DNA replication system of Li and Kelly (1984) using HeLa cell extracts, immunoaffinity-purified T antigen (Simanis and Lane 1985), and immunopurified p53 protein (Wade-Evans and Jenkins 1985). Figure 1 (top left) shows the results of in vitro replication assays performed in the absence or presence of purified *dl*162p53 and *dl*518p53. *dl*162p53 (Jenkins et al. 1985) is an internal deletion mutant of mouse p53 that binds to T antigen and inhibits SV40 DNA replication in vivo (Braithwaite et al. 1987; Stürzbecher et al. 1988c); *dl*518p53 (Jenkins et al. 1985) is a noninhibiting hsp72/73-binding mouse p53 mutant (Braithwaite et al. 1987; Stürzbecher et al. 1988c). In the presence of T antigen, SV40 DNA is an efficient template for replication synthesis, whereas no replication occurs with an SV40 origin minus plasmid, confirming the specificity of the system. Addition of 100 ng of *dl*162p53 protein almost completely blocks SV40 DNA replication. In contrast, introducing 100 ng of *dl*518p53 into the reaction has little effect on DNA replication. Thus, the suppressor effect of mouse p53 on SV40 DNA replication can be reconstituted in vitro, and this activity is restricted to T-antigen-binding-competent p53 as it is in vivo. This result is consistent with a model in which p53 exerts its effect in replication assays by direct interaction with T antigen and associated components of replication rather than by some secondary mechanism such as (say) p53-directed changes in cellular gene expression. During SV40 DNA replication, specific binding of T antigen to the SV40 origin of replication is absolutely required to start the synthesis of each daughter DNA molecule (Margolskee and Nathans 1984; Cole et al. 1986; Deb et al. 1986a; Li et al. 1986; Paucha et al. 1986). T antigen catalyzes unwinding of circular SV40ori$^+$ duplex DNA and subsequently acts as a helicase to unwind the DNA helix in advance of the replication fork (Stahl et al. 1986; Dean et al. 1987; Goetz et al. 1988; Wiekowski et al. 1988).

To test whether the inhibition of SV40 DNA replication by mouse p53 was due to sequestering T antigen from its binding sites at the origin of replication, we performed nitrocellulose filter binding assays using a ^{32}P-end-labeled 65-bp DNA fragment encompassing the SV40 minimal origin of replication (T-antigen-binding site II). Addition of up to 250 ng of *dl*518p53 does not perturb T-antigen binding to site II. (Fig. 1, top right). In contrast, presence of more than 200 ng of *dl*162p53 causes a moderate reduction in site II binding. Thus, the observed reduction of T-antigen binding caused by *dl*162p53 is not sufficient to explain its suppression of SV40 DNA replication. Next, we determined T-antigen helicase activity in the absence or presence of purified *dl*162p53 or *dl*518p53. As substrate of DNA helicase, we used single-stranded M13mp18 DNA, carrying an annealed ^{32}P-labeled 19-mer oligonucleotide. The result is shown in Figure 1 (bottom). With increasing amounts of *dl*162p53 present in the reaction, T-antigen helicase activity was blocked some 65%, whereas *dl*518p53 did not affect the T-antigen-catalyzed release of base-paired 19-mer oligonucleotide from M13mp18 DNA. Neither *dl*162p53 nor *dl*518p53 exhibited any helicase activity by itself. From these findings, we conclude that suppression of SV40 DNA replication by mouse p53 is at least partially caused by interference with T-antigen helicase activity.

A variety of point mutants of mouse p53 exhibit altered activity in SV40 DNA replication assays

To gain a more detailed understanding of the structural requirements for p53-mediated inhibition of SV40 replication, we constructed a mouse p53 expression library of C→T transition mutants by methoxylamine treatment of plus-strand DNA in M13 followed by transfer of mutagenized plus-strand/wild-type minus-strand heteroduplexes into the SV40 origin-containing vector pBC12CMV (Cullen 1986). This library was screened for the presence of p53 mutants with novel replication phenotype. Plasmid DNA grown up from individual clones of transfected *E. coli* DH5 was assayed for DNA replication by transient expression in COS cells, as we described elsewhere (Braithwaite et al. 1987; Stürzbecher et al. 1988c). Figure 2 (top right) gives a summary of these studies for different mutant p53 constructs (Fig. 2, top left). The data represent, in each case, the mean value of at least 35 separate bacterial plate transfections. Although wild-type mouse p53 (CMVmsp53) suppresses plasmid replication to about 6% of the non-p53-expressing control plasmid (CMVIL2), all the plasmids expressing these repre-

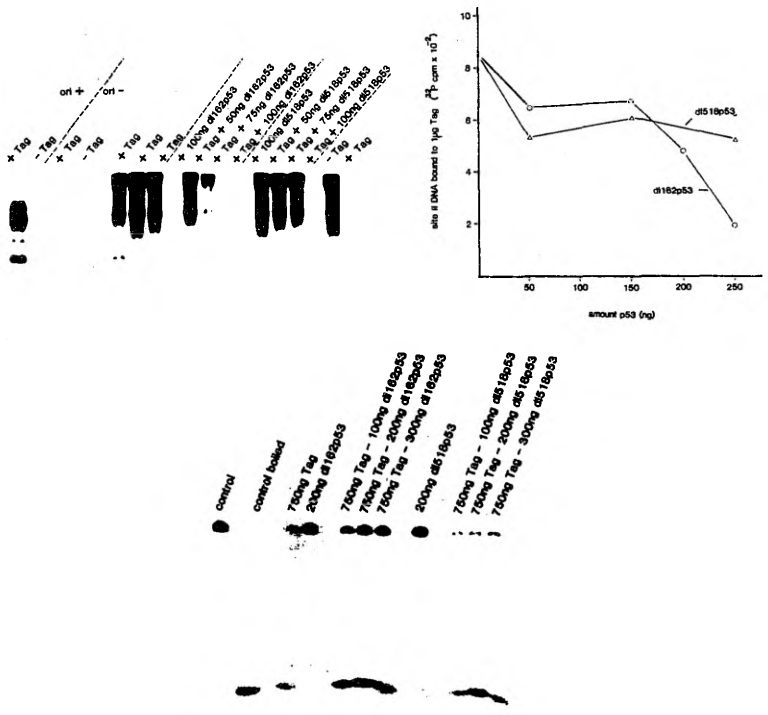

Figure 1 Activity of immunopurified p53 proteins in vitro. (*Top left*) Inhibition of SV40 DNA replication following addition of T-antigen (Tag)-binding-competent mouse p53 protein to a Li and Kelly (1984) in vitro replication system; (*top right*) inhibition of T-antigen SV40 origin binding by mouse p53; (*bottom*) inhibition of T-antigen intrinsic helicase activity by mouse p53.

sentative mutant p53 proteins inhibit either poorly or not at all.

Biochemical analysis of the mutant proteins revealed that they fall into three classes with different properties. Figure 2 (bottom left) shows immunoprecipitations of [^{35}S]methionine-labeled mutant p53 proteins from transfected COS cells. Mutant 8 protein is representative of the first class of mutants. Mutant 8 p53 retains the ability to complex with T antigen, since it coprecipitates with the anti-T-antigen monoclonal antibody PAb 423 (Harlow et al. 1981). An exclusively nuclear subset of mutant 8 protein expresses the epitope recognized by antibody PAb 246 (Yewdell et al. 1986), which in previous work has been found to correlate with T-antigen binding (Milner and Cook 1986; Yewdell et al. 1986); another subpopulation, distributed in both nucleus and cytoplasm, precipitates in complex with hsp72/73 (Fig. 2, bottom left). DNA sequence analysis reveals that mutant 8 has acquired a point mutation at amino acid residue 274, changing proline to leucine. Residue 274 lies within one of the regions of primary amino acid sequence implicated in stable complex formation with T antigen (Jenkins et al. 1988), suggesting that this mutant protein is defective in replication suppression because the change in residue 274 in some way directly compromises, but does not abolish, T-antigen binding.

Immunoprecipitation of labeled mutant 4 protein (Fig. 2, bottom left), which is representative of a second

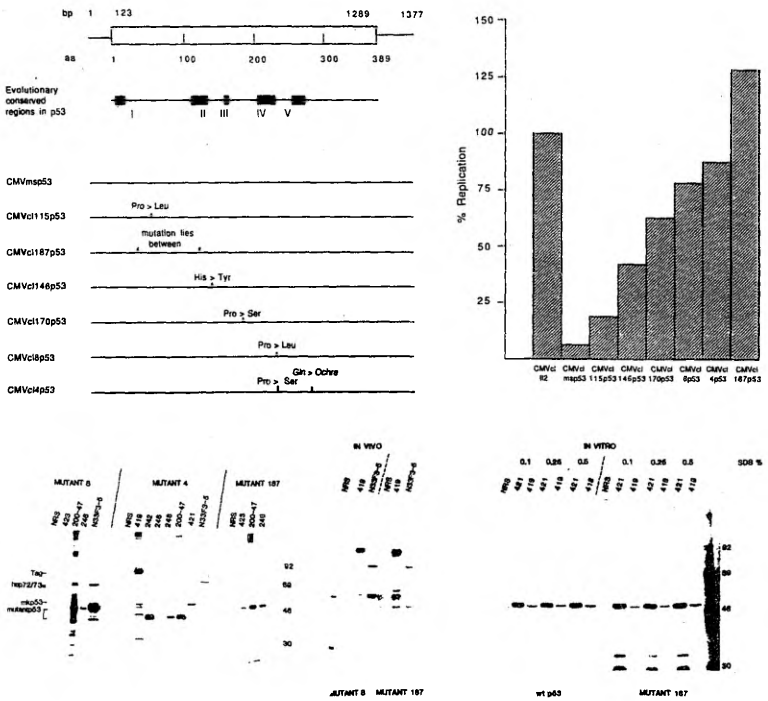

Figure 2 Murine p53 mutant proteins with altered replication phenotype. (*Top left*) Cartoon of mutant cDNA sequence showing positions and structures of mutations; (*top right*) histogram of mouse p53 mutant replication phenotypes; (*bottom left*) in vivo association of mouse mutant p53 proteins with either T antigen or heat-shock-related proteins hsp 72/73 in SV40-transformed COS cells; (*bottom right*) in vitro association assay of T antigen/p53 binding.

distinct class of suppressor-defective mutants, shows a polypeptide immunoreactive with anti-mouse p53 monoclonal antibodies PAb 242, 246, 248, and 200-47, but not with PAb 421, which has increased electrophoretic mobility on SDS-PAGE gels and binds neither T antigen nor hsp72/73. DNA sequence analysis reveals that mutant 4 has acquired two separate mutations (Fig. 2, top left). The first, like mutant 8, alters residue 274 but in this case results in a proline to serine change. The second involves residue 314, giving rise to the glutamic acid to ochre change. This ochre mutation explains the absence of PAb 421 reactivity, since the PAb 421 epitope lies carboxy-terminal of residue 314 between residues 370 and 378 (Wade-Evans and Jenkins 1985).

Since the carboxy-terminal region of p53 is nonessential for T-antigen binding (Jenkins et al. 1988), it is highly likely that the residue 274 lesion is solely responsible for the replication phenotype of mutant 4, in this case abolishing rather than reducing T-antigen binding.

Mutant 187 is representative of the third class of p53 mutants. Immunoprecipitation (Fig. 2, bottom) shows that mutant 187 protein binds to T antigen, expresses the PAb 246 epitope, does not complex with hsp72/73 (Fig. 2, bottom), and is exclusively nuclear (data not shown). Subfragment mapping data localize the lesion in mutant 187 as being amino-terminal of amino acid residue 157, which places it upstream of the sequence blocks implicated in T-antigen binding (Jenkins et al.

1988). Analysis of mutant 187p53–T-antigen complex stability by in vitro association and incremental SDS concentration washes (Fig. 2D) shows no decrease in stability compared to wild-type mouse p53-T antigen. Thus, we conclude that T-antigen binding, although necessary, is insufficient for p53-mediated suppression of SV40 DNA replication and that a second distinct activity, unrelated to T-antigen-binding affinity, with sequence requirements in the amino-terminal region of p53, is implicated in suppression.

Positive and negative modulation of SV40 DNA replication by human p53

In earlier reports (Braithwaite et al. 1987; Stürzbecher et al. 1988c), we showed that murine, but not human, p53 proteins suppressed SV40 DNA replication in monkey COS cells. Reasoning that this might indicate a functional role for p53 in DNA replication, we attempted to identify human p53 mutants of novel replication phenotype.

Two of the (several) phosphorylation sites on mouse p53 have been mapped to Ser-389 and Ser-312 (Samad et al. 1986; Meek and Eckhart 1988). Both these residues, whose identities and contexts are conserved between mouse, human, and *Xenopus* sp. p53 (Soussi et al. 1987) are relatively overphosphorylated in transformed mouse cells (Samad et al. 1986; Meek and Eckhart 1988). The presence of phosphate on Ser-389 of mouse p53 is due to covalent linkage of this residue with a small RNA (Samad et al. 1986).

We constructed mutant human p53 cDNA genes in which the serine residues corresponding to phosphoserine 312 and 389 on mouse p53 were converted to either alanine or isoleucine in the human protein ($hup315_{ala}$; $hup392_{ile}$) (Fig. 3, top left). We assayed these mutant p53 genes for their effect on SV40 replication in COS cells as described above. Figure 3 (top right) shows that, as before (Braithwaite et al. 1987; Stürzbecher et al. 1988c), wild-type human p53 expression plasmid CMVhup53 replicates well in COS cells, whereas replication of an equivalent plasmid expressing mouse p53 (CMVmsp53) is depressed to some 20-fold. Both $CMVhup315_{ala}$ and $CMVhup392_{ile}$ replicated with an efficiency substantially below that of CMVhup53. Cotransfection of $CMVhup315_{ala}$ or $CMVhup392_{ile}$ with a pBC12CMV vector containing an irrelevant gene sequence showed that inhibition of DNA replication operated in *trans* (data not shown). The mutations in $CMVhup315_{ala}$ and $CMVhup392_{ile}$ lie outside conserved regions III, IV, and V of p53 primary amino acid sequence (Soussi et al. 1987) that are implicated in binding T antigen (Fig. 3, top left; Jenkins et al. 1988). To test whether the mutant p53 proteins were competent to bind T antigen, in vitro association assays were carried out using [^{35}S]methionine-labeled human wild-type or mutant p53 proteins derived from cRNA-directed reticulocyte lysates (Wade-Evans and Jenkins 1985; Jenkins et al. 1988; Stürzbecher et al. 1988b) and immunoaffinity-purified T antigen (Simanis and Lane 1985). Both $CMVhup315_{ala}$ and $CMVhup392_{ile}$ p53 proteins coprecipitate in complex with T antigen indistinguishably from wild-type human p53 (Fig. 3, bottom left). Pulse-chase experiments revealed that both mutant p53 proteins have a $t_{1/2}$ similar to wild-type human p53 (data not shown), and sucrose gradient sedimentation analysis showed that these mutants assemble into higher order oligomeric forms and in complex with T antigen (Fig. 3, bottom right). Incremental detergent washes show that these mutant p53–T-antigen complexes do not exhibit increased stability over wild-type human p53–T-antigen (data not shown). From these findings, we conclude that the mutant p53 proteins encoded by $CMVhup315_{ala}$ and $CMVhup392_{ile}$ are able to suppress SV40 origin-directed DNA replication in vivo by a mechanism unrelated to T-antigen-binding affinity.

591 Ins is a mouse mutant p53 cDNA gene which, as we showed in an earlier report (Jenkins et al. 1985), gave the most vigorous response of all the mouse mutant p53 constructs we tested in rodent cell transformation assays. We therefore reconstructed the 591 Ins mutation into the human p53 cDNA gene context and tested this human p53 analog (CMVhuins615) for its replication phenotype in COS cells. Figure 3 (top right) shows the result. CMVhuins615 reproducibly overreplicates > 2.5-fold compared to CMVhup53. In vitro association assays confirm that the CMVhuins615 p53 gene product can complex with T antigen (Fig. 3, bottom left) and assemble into oligomeric forms (Fig. 3, bottom right). Only a minor subpopulation of this mutant p53 protein binds to hsp72/73 in vivo (data not shown). The ability of CMVhuins615 p53 to stimulate SV40-origin-directed episomal replication argues strongly for a direct role of p53 in the enzymology of viral DNA replication, since introduction of mutations into regions of the p53 polypeptide can cause either inhibition or stimulation of DNA synthesis.

Discussion

We are attempting to identify the normal functions of p53 and to understand the molecular basis of how abnormalities of p53 expression could contribute to a more general loss of cellular growth control. Of particular interest to us is human p53 and its biological and biochemical properties when expressed in primate systems, and as one experimental approach, we have chosen to investigate the functional relationship between p53 and SV40 T antigen and the impact of xenogeneic and mutant p53 proteins on SV40 DNA replication.

As we show here, murine p53 is able to suppress replication from the SV40 origin in a cell-free system, an observation that supports the notion that such suppression involves p53 interacting directly with T antigen and associated factors rather than by "action at a distance" such as modulating some aspect of gene expression.

However, since in many cases transcription and replication are closely linked (for review, see DePamphilis 1988), we cannot exclude the possibility that p53 has additional transcriptional regulation properties. The in

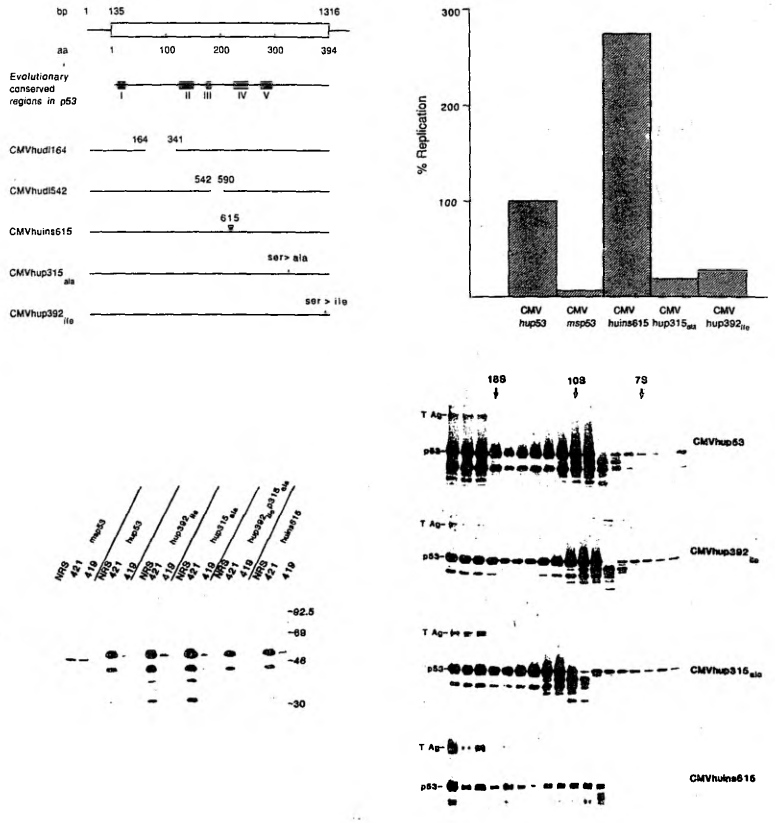

Figure 3 Characterization of human p53 mutants with novel replication phenotypes. (*Top left*) Cartoon of mutant p53 sequences showing positions and structures of mutations; (*top right*) histogram of human mutant p53 replication phenotypes; (*bottom left*) in vivo association of human mutant p53 proteins with T antigen in SV40-transformed COS cells; (*bottom right*) sucrose gradient analysis of human mutant p53/T antigen oligomers from SV40-transformed COS cells.

vitro data suggest that mouse p53 interferes with some event either at, or temporally close to, the initiation of leading-strand synthesis rather than during elongation or deconcatenation, since there is no evidence in vitro for preferential labeling of restriction fragments at or near *ori* (Stürzbecher et al. 1988b). Site-specific DNA-binding studies (Fig. 1) showed that mouse p53 could somewhat reduce T-antigen binding to the origin of replication (site II). Mouse p53 also reduced detectable T-antigen helicase activity (Fig. 1), albeit at ~5× greater concentration of p53 protein than that required for equivalent suppression of DNA replication in vitro. Analysis of mouse p53 mutant 187 indicated that the relatively high affinity of mouse p53 as opposed to

human p53 for T antigen was not directly associated with replication suppression, since 187 protein possessed comparable T-antigen binding to wild-type mouse p53 (Fig. 2, bottom right), yet did not inhibit SV40 replication in vivo (Fig. 2, top right).

The results obtained with human p53 mutants CMVhup53 315_{ala}, CMVhup53 392_{ile}, and CMVhup53 Ins 615 argue strongly for a direct role of p53 in the SV40 replication complex and, by analogy, with some equivalent component of cellular DNA replication/repair synthesis. Whether or not mutations perturbing the replication phenotype of p53 are present in instances of human neoplasia remains to be seen. However, it is apparent that studies of p53 gene abnormality in human tumor material should include screening for the presence of point mutations as well as gross gene rearrangement or deletion, since as we predicted earlier (Jenkins et al. 1985) and demonstrate here, point mutation and minor sequence change can dramatically alter the biological properties of the encoded human oncoprotein. It is also clear that, although in at least one experimental system loss of p53 expression appears to be coordinate with acquisition of the transformed phenotype (Rovinski et al. 1987), in the in vivo replication system we describe in this report, mutant p53 proteins can clearly exert a transdominant effect in the presence of wild-type p53 protein—in this case COS cell p53—probably by competing the normal protein from its appropriate binding site on T-antigen protein and thus abrogating the "normal" function of the unmutated p53 protein. It has been suggested by S. Benchimol (International p53 Workshop 1988) that p53 may be a suppressor or "antioncogene" protein. We propose that such a process of displacement could allow a mutant suppressor gene to act in some cases in a dominant fashion.

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p53 interacts with p34^{cdc2} in mammalian cells: implications for cell cycle control and oncogenesis

Horst-Werner Stürzbecher[1], Toivo Maimets[1,2], Peter Chumakov[1,3], Ruth Brain[1], Christine Addison[1], Viesturs Simanis[4], Karran Rudge[1], Robin Philp[1], Margaret Grimaldi[1], Will Court[1] & John R. Jenkins[1]

[1]*Cell Proliferation Laboratory, Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, UK;* [2]*Estonian Biocenter, Riietorn, Toomemägi, Tartu, Estonia 202400, USSR;* [3]*Institute of Molecular Biology, Academy of Sciences of the USSR, Vavilov Street 32, Moscow, USSR;* [4]*Institute Suisse de Recherches Expérimentales sur le Cancer, Chemin des Boveresses 155, CH-1066 Epalinges s/Lausanne, Switzerland*

The p53 gene product has been implicated in both human and animal tumorigenesis. p53 complexes with the transforming proteins encoded by several different DNA tumour viruses. We demonstrate that human p53 is phosphorylated by the mammalian p34^{cdc2} kinase *in vitro* and coprecipitates with p34^{cdc2} *in vivo*. Our observations suggest that phosphorylation of p53 by p34^{cdc2} kinase may regulate the known activities of p53 in the initiation steps of DNA replication in mammalian cells.

Introduction

The progression of cells from normal proliferative control to malignancy appears to require a number of distinct events that can be temporally and functionally separate. An increasing body of evidence suggests that the DNA tumour viruses, many of which, such as Epstein–Barr virus, hepatitis B virus and the human papilloma viruses type 16, 18 and 33, are closely associated with specific human malignancies, exert their carcinogenic potential via direct physical association of viral transforming gene products with multiple cellular protein targets. In contrast to the oncogenic retroviruses, DNA tumour virus oncoproteins appear to lack structurally related cellular counterparts. However, in several cases where cellular binding targets are known, these cellular proteins are involved in growth regulation. Examples include polyoma virus middle T antigen which binds cellular *src* (c-*src*) (Courtneidge & Smith, 1983) and the adenovirus E1A proteins which interact with as many as ten cellular proteins including the retinoblastoma susceptibility gene product (Rb-1) (Whyte *et al.*, 1988). In untransformed cells, proto-oncogenes presumably interact with cellular factors which are functional homologues of such viral proteins and it is clear that a search for these cellular factors is likely to reveal components of the mechanisms that control cell proliferation.

The best characterised of such viral/host protein systems is the Large tumour antigen (T antigen) of Simian Virus 40 (SV40), which forms specific complexes with several cellular proteins including DNA polymerase α (Smale & Tjian, 1986), RB-1 (DeCaprio *et al.*, 1988) whose function remains obscure, and p53 which was itself first identified in complex with T antigen (Lane & Crawford, 1979).

The T antigen/p53 complex has been studied in some detail (for recent review see Jenkins & Stürzbecher, 1988). This complex assembles using highly conserved domains of the p53 protein (Jenkins *et al.*, 1988), and appears to be involved in the recruitment of viral DNA replication origins (Stürzbecher *et al.*, 1988; Wang *et al.*, 1989).

p53 can itself act as a dominant transforming gene (see Jenkins & Stürzbecher, 1988) and displays at least two distinct and separable biological activities in classical transfection assays (Jenkins *et al.*, 1985); cellular immortalisation (Jenkins *et al.*, 1984b; Jenkins *et al.*, 1985) and co-operation with an activated *ras* gene (Jenkins *et al.*, 1984b; Eliyahu *et al.*, 1984; Parada *et al.*, 1984). Some mutant p53 proteins have greatly enhanced activity in such transformation assays indicating that p53 can be activated by mutations (Jenkins *et al.*, 1985; Finlay *et al.*, 1988). Intriguingly, a second line of evidence suggests that p53 may act as a 'recessive oncogene' or 'tumour suppressor gene'. Loss or rearrangement and mutation of both p53 alleles is a common event in murine erythroleukemias induced by Friend virus (Mowat *et al.*, 1985) and it has been proposed by Benchimol that loss of wild-type p53 expression is an obligate step in Friend virus mediated transformation. Mutant p53 genes have been identified in human colorectal tumour xenografts in which the second p53 allele is absent (Baker *et al.*, 1989) and also in human lung tumours (Takahashi *et al.*, 1989). In either event the activities of p53 as oncogene or anti-oncogene product are likely to impact upon the cell via interactions with other cellular proteins of which some may be either functional analogues of the p53-binding viral gene products, or competitors with such products for complex formation with p53. One such candidate protein is a polypeptide of approx. 34 Kilo daltons mol. weight which has been reported to coprecipitate with p53 derived from mouse cell lines (Milner *et al.*, 1989).

cdc2 was first defined in the fission yeast *S. pombe* (Nurse, 1985; Hayles & Nurse, 1986) and genetic data from this experimental system have defined two distinct cell cycle control events which require the cdc2 gene product. The first, called start, is located in late G_1 and marks the commitment of cells to the mitotic cycle (Nurse & Bissett, 1981). The second event is located in late G_2 and marks the initiation of mitosis (Nurse, 1975; Thuriaux *et al.*, 1978). p34^{cdc2} is the mammalian homologue of *S. pombe* cdc2 (Dunphy *et al.*, 1988). The

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two proteins share 63% structural homology and $p34^{cdc2}$ will complement mutations in the cdc2 gene of *S. pombe* (Lee & Nurse, 1987). In higher eukaryotes there are no genetic data on $p34^{cdc2}$ functions but a body of evidence implicates $p34^{cdc2}$ in the action of Maturation Promoting Factor (MPF) at the G_2 to mitosis transition ($G_2 \rightarrow M$) (Dunphy *et al.*, 1988) and specifically as the catalytic subunit of the MPF associated Serine/Threonine histone H1 kinase (Arion *et al.*, 1988). Here we report that human p53 is phosphorylated *in vitro* by $p34^{cdc2}$ protein kinase and that minor but detectable amounts of p53 and $p34^{cdc2}$ proteins copurify in immune precipitations. Our observations link p53 with a known component of eukaryotic cell cycle control and suggest that such $p53/p34^{cdc2}$ interactions may play a role in the control of cellular DNA replication.

Results

$p53/p34^{cdc2}$ association

In earlier experiments in monkey COS cells we have observed that immunoprecipitations using antibodies reactive against p53 revealed additional protein species migrating with an approximate M_r of 34 Kd while antibodies reactive against $p34^{cdc2}$ coprecipitated multiple protein species of M_r range between 50–60 Kd. It is known that $p34^{cdc2}$ both phosphorylates *in vitro* and forms specific complexes *in vivo* with both the 62 Kd Cyclin B protein (Pines & Hunter, 1989) and another protein of 60 Kd (Giordano *et al.*, 1989) and we reasoned that another target might be p53. We were unable to generate adequate N-Chlorosuccinimide cleavage maps of either the 34 Kd protein or a 53 Kd species which appeared to coprecipitate in anti $p34^{cdc2}$ immunoprecipitations due to cross-contamination between multiple polypeptide species comigrating with this region of the gel. We therefore carried out transfections into COS cells of a mutant human p53 cDNA construct, hudl164, encoding a p53 protein with higher mobility on SDS gels. This mutant cDNA gene (hudl164), is the structural homolog of mouse p53 mutant dl162 (Jenkins *et al.*, 1985). hudl164 protein migrates as a lower molecular weight species of 43 Kd and this circumvents possible contamination from 50–60 Kd species. However, although truncated, hudl164 mutant p53 retains the ability to bind T antigen (Jenkins *et al.*, 1988), and to self oligomerise (H.-W. Stürzbecher, unpublished) and unlike many other p53 mutant proteins is located in the cell nucleus (Stürzbecher *et al.*, 1987). To exclude the possibility that these proteins might coprecipitate merely because they are each separately complex to T antigen, we first carried out a cascade immunoprecipitation with the T antigen reactive monoclonal antibody PAb419 (Harlow *et al.*, 1981). We followed this by one immunoprecipitation each with the two anti $p34^{cdc2}$ sera cdc2A and cdc2B, and finally with an immunoprecipitation using the p53 reactive monoclonal antibody PAb421 (Harlow *et al.*, 1981). Figure 1a shows the results. Three sequential precipitations with anti-T antigen antibody depleted T antigen and T antigen bound hudl164 protein from the lysate and the second and third stages of this cascade exhibited increased amounts of the cellular PAb419 cross-reactive 35 Kd species (Crawford *et al.*, 1982) for which PAb419 is known to have lower affinity. Subsequent immunoprecipitations from the T antigen depleted lysate using anti $p34^{cdc2}$ sera reactive with two different peptide sequences (Figure 1a) demonstrated that in both cases a novel 43 Kd polypeptide now coprecipitated with $p34^{cdc2}$ and that the 43 Kd species comigrated with the hudl164 gene product.

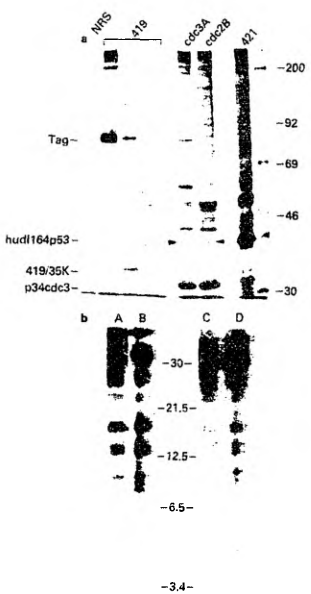

Figure 1 p53 interacts with $p34^{cdc2}$ *in vivo*. **(a)** Cascade immunoprecipitation of [^{35}S]methionine labelled CMVhudl164/CMvhucdc2 double-transfected COS cells. Cleared lysate was *sequentially* immunoprecipitated three times with monoclonal anti SV40 large T antigen antibody PAb419 (Harlow *et al.*, 1981) by cdc2A and cdc2B anti $p34^{cdc2}$ peptide antisera (cdc2A was raised against a peptide corresponding to the PSTAIR motif in cdc2 protein and cdc2B against the carboxyl terminus of human $p34^{cdc2}$ (Draetta & Beach, 1988)) and monoclonal anti p53 antibody PAb421 (Harlow *et al.*, 1981). NRS: normal rabbit serum. 419/35K: 35 Kd protein, cross-reactive with PAb419 (Harlow *et al.*, 1981; Crawford *et al.*, 1982). **(b)** N-Chlorosuccinimide cleavage of proteins from CMVhudl164/CMVhucdc2 double-transfected [^{35}S]-labelled COS cells. (A/C) Cleavage pattern of hudl164p53 coprecipitated in immunoprecipitate using antiserum cdc2B. (B/D) Cleavage pattern of hudl164p53 from PAb421 precipitate. A + B and C + D are derived from independent experiments

We carried out N-Chlorosuccinimide cleavage comparisons between the 43 Kd polypeptide coprecipitating with the $p34^{cdc2}$ reactive sera and bonafide hudl164 protein. The results are shown in Figure 1b and demonstrate that the coprecipitating 43 Kd species and hudl164 protein are indistinguishable. Immuno-

precipitations from p53 expression construct transfected monkey COS cell lysates using either anti-p53 or anti-$p34^{cdc2}$ antibodies, followed by Western blotting using anti-p53 monoclonal PAb421 (Harlow *et al.*, 1981) or the cdc2B anti-$p34^{cdc2}$ antiserum, revealed that anti-p53 monoclonal antibody PAb421 coprecipitates a minor but detectable fraction of anti-$p34^{cdc2}$ cross-reactive material of appropriate molecular weight (data not shown) and we conclude that a subpopulation of p53 and $p34^{cdc2}$ proteins appear to copurify from monkey COS cells. We note (Figure 1a) that we are unable to detect any corresponding 34 Kd species in anti-T antigen immunoprecipitations using monoclonal antibody PAb419 (of the same class and subclass as PAb421) suggesting that coprecipitation of $p34^{cdc2}$ and p53 is due neither to unspecific immunoglobulin 'stickyness' nor to some trimeric complex between $p34^{cdc2}$, T antigen and p53.

Phosphorylation by cdc2 kinase

We reasoned that the apparent association between p53 and $p34^{cdc2}$ might result from some enzyme/substrate relationship and we checked the p53 protein sequence for possible substrate motifs. We identified two regions that were candidates for phosphorylation by $p34^{cdc2}$ kinase, one of which corresponds to a site known to be phosphorylated *in vivo* and relatively over-phosphorylated in transformed cells (Samad *et al.*, 1986; Meek & Eckhardt, 1988). Peptide 1 has homology with a site on T antigen known to be phosphorylated *in vitro* by $p34^{cdc2}$ kinase with a resulting stimulation of origin binding activity (McVey *et al.*, 1989) (Table 1). We synthesised 2 peptides corresponding to these putative target sites. Peptide 1 consisted of three linear repeats of the sequence NTSSSPQPY corresponding to residues 311 to 318 while peptide 2 consisted of three linear repeats of the sequence PLSSSVPSY corresponding to residues 92–99 in human p53. We carried out *in vitro* kinase assays on these peptides using immunoprecipitations of $p34^{cdc2}$ kinase from asynchronous HeLa cell cultures. In the absence of any exogenous substrate a coprecipitating protein of around 60 Kd, most probably cyclin B (Pines & Hunter, 1989) serves as phosphoacceptor in anti-$p34^{cdc2}$ immunoprecipitates. Transfer of radiolabeled phosphate to added histone H1 confirms that antiserum cdc2B recognizes $p34^{cdc2}$ (Figure 2a). The results of kinase assays with peptides 1 and 2 as exogenous substrates are shown in Figure 2b. Neither peptide was phosphorylated by control serum precipitations, including one serum (JR4K) which precipitates a kinase activity distinct from $p34^{cdc2}$ kinase. However peptide 1 was significantly phosphorylated in the presence of $p34^{cdc2}$ kinase immunoprecipitated by serum cdc2B (Figure 2b). In control experiments we found that the immunising peptide used to raise serum cdc2B competed $p34^{cdc2}$ kinase activity from cdc2B immunoprecipitations, confirming the identity of this activity as $p34^{cdc2}$ kinase (data not shown). To further investigate the possibility that p53 is a substrate for $p34^{cdc2}$ kinase we immunopurified human p53 protein from insect cells infected with an appropriate recombinant baculovirus vector, and tested the purified protein as a $p34^{cdc2}$ kinase substrate. Human p53 protein was purified by immunoaffinity chromatography on protein A-sepharose coupled PAb421 monoclonal antibody. In order to avoid gross pH changes and potential contamination with antibody, p53 protein was eluted with excess peptide corresponding to the PAb421 epitope (Wade-Evans & Jenkins, 1985). Figure 2C shows the result of *in vitro* kinase assays. As has been published by others (Jay *et al.*, 1981), immunoprecipitated p53 alone exhibits a weak associated kinase activity. In the presence of added $p34^{cdc2}$ kinase immunoprecipitated from HeLa cells however there is a substantial increase in the phosphorylation of p53. We conclude from these results that p53 is a substrate of the $p34^{cdc2}$ kinase *in vitro*.

To identify which serine residue in the peptide 1 kinase motif was utilised as phosphate acceptor, we synthesised three variants of peptide 1 in which two out of the three serine residues were replaced by alanine. Of these variants, the peptide retaining the serine corresponding to serine 315 in human p53 was utilised efficiently as a substrate by $p34^{cdc2}$ kinase while the peptides retaining serines 313 or 314 were not (Figure 2D). We conclude that serine 315 within this motif is specifically phosphorylated by $p34^{cdc2}$ kinase.

Cell cycle specificity

Two distinct subpopulations of $p34^{cdc2}$ kinase are detectable in HeLa extracts. One consists of $p34^{cdc2}$ in complex with Cyclin B (p62/cdc2) and is maximally active during mitosis (Pines & Hunter, 1989; Draetta & Beach, 1988). The other is comprised of $p34^{cdc2}$ in complex with a polypeptide of approximately 60 Kd (p60/$p34^{cdc2}$) and is reportedly active in interphase (Giordano *et al.*, 1989). To determine whether or not peptide 1 sequence was utilised as substrate in a cell cycle restricted manner, we prepared cell cycle stage specific extracts from a centrifugally elutriated HeLa cell population. Anti-$p34^{cdc2}$ immunoprecipitations from these extracts were then assayed for their ability to utilise either peptide 1 or histone H1 as a kinase substrate. As can be seen from Figure 3, the $p34^{cdc2}$ kinase activity responsible for phosphorylation of peptide 1 is cell cycle restricted, with an increase in detectable activity in fractions relatively enriched for S → G2 cells. Both peptide 1 and histone H1 are utilised as substrates. Analysis of cell population enriched for G2-M cells revealed that kinase activity continues to increase into G2-M. Once again both peptide 1 and histone H1 as well as immunoaffinity purified p53 are utilised as substrates (data not shown). We conclude from this that

Table 1 $p34^{cdc2}$ kinase recognition motifs in SV40 T antigen and p53

Protein	*aa-Sequence*	*Reference*
SV40 T antigen	120-S Q H S T P P K-127	McVey *et al*, 1989
Human p53	311-N T S S S P Q P-318	Harlow *et al*, 1985
Monkey p53	311-N T S S S P Q P-318	Rigandy and Eckhart, 1989
Mouse p53	308-C T S A S P P Q-315	Jenkins *et al*, 1984a
Rat p53	309-S T S S S P Q Q-316	Soussi *et al*, 1988

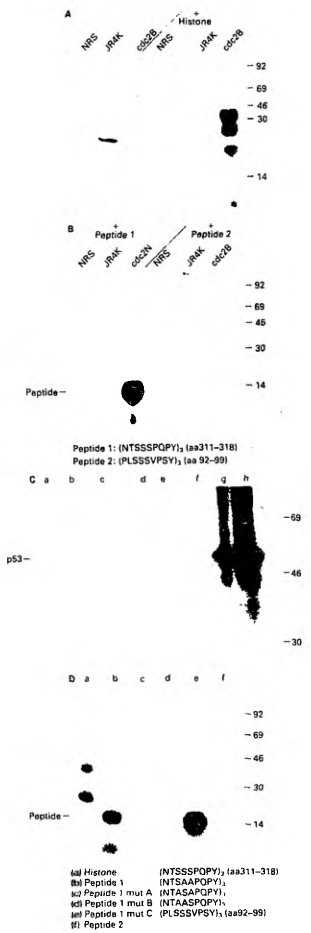

Figure 2 $p34^{cdc2}$ kinase phosphorylates p53 *in vitro*. (**A**) Control immune precipitations with or without *H*istone H1 as exogenous substrate. (**B**) With peptides corresponding to potential $p34^{cdc2}$ kinase target sites in human p53. NRS: Normal rabbit serum. *JR4K: Polyclonal rabbit antiserum* recognizing an irrelevant kinase activity (details to be published elsewhere). cdc2B: Rabbit antiserum raised against a peptide corresponding to the carboxyl terminus of human $p34^{cdc2}$ (Draetta & Beach, 1988). (**C**) With immunoaffinity purified human p53 produced in recombinant *baculovirus infected cells as exogenous substrate*. **a–e**: NRS Immunoprecipitates. **f–h**: cdc2B Immunoprecipitates. **b, c, g, h**: Immunoaffinity purified human p53 as substrate. **d, e**: Kinase assay on

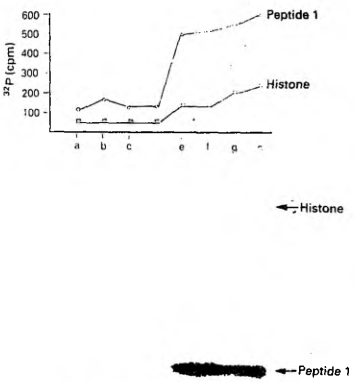

Figure 3 *Phosphorylation of* p53 peptide 1 by $p34^{cdc2}$ kinase is cell cycle regulated. Protein kinase assays of cdc2B anti-peptide serum precipitates from HeLa cells separated by elutriation centrifugation (fractions **a** to **h**) using Histone H1 or p53 peptide 1 (see Figure 2) as substrates. [$^{32}PO_4$] incorporation into histone H1 and peptide 1 are shown at the top, percentage of cells in *G1*, S-phase and G2 in each elutriated fraction, determined by flow-cytometry, is shown at the bottom

p53 is a potential $p34^{cdc2}$ kinase substrate from the onset of S-phase.

Biochemical characterization

To determine whether alterations at this potential $p34^{cdc2}$ kinase site alter the biochemical properties of p53, we constructed a mutant p53 cDNA gene ($hup315_{ala}$) in which serine 315 was replaced by alanine, and tested this mutant for its effects on T antigen binding and oligomerization in COS cells. Secondary structure predictions suggested that this substitution would have minimal effects on local structure. We transfected COS cells with an expression vector expressing $hup315_{ala}$ and analysed the biochemical properties of the mutant protein. Figure 4 shows the results. Sucrose gradient sedimentation and immune precipitation analysis showed that $hup315_{ala}$ protein enters the characteristic hetero-oligomeric complexes with T antigen, cosediments with T antigen (Figure 4a) and, unlike many mutant p53 proteins (Sturzbecher *et al.*, 1987), does not complex detectably with the heat shock related proteins hsp 72/73 (Figure 4a and data not

immunoaffinity purified human p53 only. **a, b, d, f, g**: Incubation time 20 min at 30°C. **c, e, h**: Incubation time 60 min at 30°C. (**D**) Identification of the phosphate acceptor amino acid in the $p34^{cdc2}$ kinase target site (as 311–318) of human p53. $p34^{cdc2}$ kinase assays performed on cdc2B anti-$p34^{cdc2}$ immunoprecipitates from HeLa cells with the indicated peptides as exogenous substrates

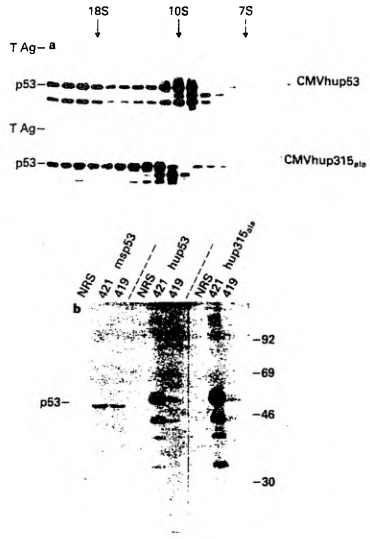

Figure 4 Mutant human p53 with altered $p34^{cdc2}$ kinase recognition motif inhibits SV40 DNA replication *in vivo*. (**a**) Sucrose density gradient analysis of $hup315_{ala}$ p53 from transfected COS cells. (**b**) *In vitro* association of $hup315_{ala}$ p53 with immunopurified SV40 large T antigen.

shown). We carried out *in vitro* association assays between immunopurified T antigen and $hup315_{ala}$ p53 protein synthesised *in vitro* in rabbit reticulocyte lysate. Figure 4b shows that $hup315_{ala}$ protein complexes with T antigen *in vitro*. In other experiments we found that $hup315_{ala}$ protein localised in the nucleus of transfected COS cells as wild-type p53 does (data not shown).

Thus, $hup315_{ala}$ protein, although mutant, retains many of the wild-type characteristics of p53 protein including the ability to participate in heterooligomers, to bind T antigen, to localise appropriately, and not to bind hsp 72/73 proteins.

We are currently investigating whether substitution of alanine for serine at position 315 compromises some replication associated function of p53 and, by inference, whether phosphorylation at this site by the $p34^{cdc2}$ kinase during S phase is obligate for this function.

Discussion

The data we present here provide evidence of interaction between the cellular oncoprotein p53, and $p34^{cdc2}$ which is likely to be the master regulator of cell cycle progression. Our experiments do not exclude the possibility that copurification is simply the result of some residual enzyme/substrate association. However, precedent exists for a combined substrate/binding target model, in that $p34^{cdc2}$ both phosphorylates *in vitro* and copurifies in complex with, the p62 Cyclin B gene product (Pines & Hunter, 1989; Draetta & Beach, 1988; Brizuela *et al.*, 1989). The *in vitro* kinase activities associated with monomeric $p34^{cdc2}$, and $p34^{cdc2}$ in complex with p62/Cyclin B show different substrate specificities (Brizuela *et al.*, 1989) and it has been reported that $p34^{cdc2}$ in complex with a cellular protein of $\sim M_r$ 60 Kd exhibits an *in vitro* histone kinase activity whose activity maximum in the cell cycle differs from $p34^{cdc2}$ in complex with p62 Cyclin B (Giordano *et al.*, 1989). Thus, a possible role for such an association between p53 and $p34^{cdc2}$ might be to confer substrate and/or cell cycle position specificity to the $p34^{cdc2}$ associated Serine/Threonine kinase.

Earlier work from a number of groups has produced somewhat contradictory evidence linking p53 with various stages of the cell cycle. Overall, the data suggest that p53 might be involved in the $G_0 \rightarrow G_1$ transition (Milner, 1984; Mercer *et al.*, 1982; Mercer *et al.*, 1984; Reich & Levine, 1984). The data indicating an involvement of $p34^{cdc2}$ in mammalian cell cycle progression are predominantly biochemical and have, to date, implicated the G_2-M transition alone as a site of action. Nevertheless, the degree of protein sequence conservation between mammalian $p34^{cdc2}$, *S. pombe* cdc2 and *S. cerevisiae* cdc28 and the observation that $p34^{cdc2}$ and cdc28 can approximate both the G_1-S and G_2-M obligate functions of cdc2 when expressed in a cdc2 defective *S. pombe* strain is suggestive that $p34^{cdc2}$ is competent to fulfil some, as yet unidentified, role at mammalian cell G_1-S. It is significant in this context that in higher eukaryotes the major cell cycle control point (Restriction point) is defined at the G_1-S boundary (Pardee, 1974; Pardee *et al.*, 1978; Zetterberg & Larsson, 1985) and that both *S. pombe* cdc2 and *S. cerevisiae* cdc28 encode genetically defined control functions obligate for progression through *start* into the onset of S phase and replicative DNA synthesis.

The use of SV40 DNA replication as a model system has indicated that p53 protein is involved in initiation functions at the onset of DNA replication and we have proposed elsewhere (Jenkins & Stürzbecher, 1988; Stürzbecher *et al.*, 1988; Jenkins *et al.*, 1989) that by analogy, p53 might be involved in concert with cellular helicases in the recruitment of cellular DNA replication origins at and beyond the $G_1 \rightarrow S$ boundary. Mouse p53 protein (SV40 cannot replicate in somatic mouse cells) blocks SV40 DNA replication both *in vivo* (Braithwaite *et al.*, 1987) and *in vitro* (Stürzbecher *et al.*, 1988; Wang *et al.*, 1989). Interestingly, mouse p53 is also able to displace DNA polymerase α from complex with T antigen (Gannon & Lane, 1987) suggesting a possible additional replication inhibitory mechanism.

Here we demonstrate that p53 is a substrate *in vitro* for the $p34^{cdc2}$ kinase and that kinase activity derives from cell populations enriched for S-phase and beyond. The peptide I substrate motif maps to a site on p53 that is phosphorylated *in vivo* and more heavily phosphorylated in transformed than in untransformed cells (Samad *et al.*, 1986; Meek & Eckhardt, 1988). An attractive interpretation of our data suggests a mechanism whereby a replication-related activity of p53 might be controlled. In this model p53 protein accumulating in G_1 would lack phosphate modification at the peptide

1 site and would be either inactive in, or an active suppressor of, origin recruitment. Upon phosphorylation by $p34^{cdc2}$ associated S phase kinase, p53 would actively participate in, or fail to suppress, the initial events of DNA replication, i.e. recognition and/or unwinding of replication origins prior to the onset of strand synthesis.

Further experiments are required to determine the precise involvement of $p34^{cdc2}$ kinase but the evidence to date suggest that p53 itself regulates the initial DNA unwinding step at the onset of eukaryotic DNA replication.

Materials and methods

Plasmids

The p53 and $p34^{cdc2}$ cDNA expression vectors used in the present study were constructed in the pBC12CMV vector described by Cullen, 1986. The construction of the hudl164 mutant human p53 cDNA gene lacking base pair 165–340 of full length p53 has been described previously (Jenkins *et al.*, 1989). CMVhucdc2 contains a full length cDNA clone of human $p34^{cdc2}$ isolated from HeLa cells (P. Chumakov, unpublished).

Cell culture and transfections

10^6 monkey COS cells (Gluzman, 1981) were cotransfected with 5 μg each of plasmids CMVhup53 or CMVhul64dl and and CMVhucdc2 using the DEAE dextran transfection procedure as we described earlier (Stürzbecher *et al.*, 1987). 72 h post transfection, cells were labelled with 500 μCi of [^{35}S]methionine (Amersham Corp.) for 4 h. The cells were then washed and lysed for 30 min on ice with 1 ml of NP40 containing buffer (100 mM Tris/HCl, pH 8.0; 100 mM NaCl; 1 mM dithiothreitol; 100 mM potassium phosphate, 10 mM sodium pyrophosphate; 50 mM β-glycerophosphate; 1 mM $ZnCl_2$; 0.5% NP40; 10% Glycerol; 0.25 mg ml^{-1} phenylmethylsulfonyl fluoride; 30 μg ml^{-1} aprotinin; Lysates were cleared by centrifugation at 105 000*g* for 30 min at 4°C. Immunoprecipitation and analysis by SDS-PAGE were carried out as described previously (Stürzbecher *et al.*, 1988).

For preparations of $p34^{cdc2}$ kinase, HeLa cells were grown in suspension culture in medium supplemented with 10% fetal calf serum and lysed by addition of 100 μl lysis buffer (50 mM Tris/HCl, pH 7.4; 0.25 M NaCl; 0.1% NP40; 5 mM EDTA; 50 mM NaF; 1 mM DTT; 60 mM β-glycerophosphate, 15 mM p-Nitrophenylphosphate; 30 μg ml^{-1} aprotinin) per 10^6 cells. After 30 min on ice, lysates were spun at 13 000*g* for 5 min and supernatants precleared with 40 μl of a 50% Protein A-agarose suspension (Pierce) and 5 μl NRS for 30 min at 4°C. Samples were incubated with antibody of 1 h at 4°C and subsequently centrifuged at 13 000*g* for 5 min. Supernatants were incubated with 40 μl Protein A-agarose for 30 min at 4°C.

Immunoprecipitates were washed 3 times with lysis buffer and once with Kinase buffer (25 mM MOPS pH 7.4; 15 mM EGTA; 15 mM $MgCl_2$; 10 mM DTT; 60 mM β-glycerophosphate; 15 mM p-Nitrophenylphosphate) prior to kinase reactions. Kinase reactions contained 500 nM cAMP-dependent protein kinase inhibitor, 15 mM γ-[^{32}P]ATP at about 1000 cpm pmol^{-1} and kinase buffer in a final volume of 50 μl. 1 mg histone H1 ml^{-1} (Sigma, type III-S); 0.3 mg ml^{-1} peptide or 0.3 mg ml^{-1} immunopurified human p53 (immunoaffinity purified as described previously—Stürzbecher *et al.*, 1988) were used as substrates in the assay. Kinase reactions were started by addition of radioactive ATP and were incubated for 20 min at 30°C. 8 μl of 5 times concentrated Laemmli sample buffer was added to stop the reaction and the samples were analysed by SDS-PAGE and autoradiography (Draetta & Beach, 1988).

Centrifugal elutriation

For centrifugal elutriation HeLa cells grown in suspension culture were washed in phosphate buffered saline (PBS), containing 0.3 nM EDTA, 1% fetal calf serum; 0.1% glucose (Draetta & Beach, 1988) and loaded onto a Beckman elutriator rotor (model JE-5.0) at 2000 rpm and at a pump rate of 6.5 ml min^{-1} at 4°C. After a 100 ml wash, 8 fractions were collected by stepwise increments of pump speed, from 8.5 to 12.5 ml min^{-1} 5×10^5 cells per fraction were taken for analysis by flow cytometry. 5×10^6 cells from each fraction were subjected to cell lysis. Equal amounts of total protein per cell extract were immunoprecipitated with cdc2B anti-peptide serum and the immunocomplexes used in the kinase assays as described in Figure 2. For flow cytometry cells were lysed in stain-detergent solution (1 g trisodium citrate, 564 mg NaCl, 300 μl NP40; 10 mg ethidium bromide in 1l H_2O; 1 mg RNAase A per 100 ml) and analysed using an argon-ion laser tuned to 488 nm (M. Ormerod, personal communication).

Partial proteolysis mapping

N-Chlorosuccimimide cleavage was performed essentially as described elsewhere (Draetta *et al.*, 1987). After analysis of [^{35}S]methionine-labelled immunoprecipitated proteins on SDS-PAGE, the wet gel was exposed to autoradiography overnight, the relevant bands were excised and rinsed several times in a 0.1% solution of urea in acetic acid and water (1 : 1). The gel slice was then exposed for 30 min to 40 mM N-Chlorosuccimimide in the same solution. After further washes the slices were equilibrated in Laemmli buffer and loaded directly onto a 17% SDS-polyacrylamide gel. The gel was fixed, dried and analysed by autoradiography.

Sucrose density gradient centrifugation: 0.9 ml of [^{35}S]methionine-labelled extract from CMVhup53 or CMVhup315_{ala} transfected COS cells were layered on top of 10 ml linear 5–20% sucrose gradients and analysed as described previously (Stürzbecher *et al.*, 1987). Gradient fractions were immunoprecipitated with PAb421. CMVhup315_{ala} encodes for a point mutation of human p53 created by oligonucleotide directed mutagenesis changing amino acid residue 315 from serine to alanine (TCT → GCT).

In vitro *association assays*

Plasmid DNAs encoding human p53 and hup315_{ala} p53 under the control of T7 promoter were transcribed *in vitro* with T7 RNA polymerase (40 mM Tris/HCl pH 7.5; 6 mM $MgCl_2$; 2 mM NaCl; 2 mM spermidine; 10 mM dithiothreitol; 100 μg ml^{-1} BSA; 0.5 mM ribonucleoside triphosphates (pH 7.0); 72 units RNasin ribonuclease inhibitor (Amersham); 10 μg linearised DNA template; 16 units T7 RNA polymerase; incubation 60 min at 40°C). Translation of cRNA products was carried out in the mRNA-dependent rabbit reticulocyte lysate system by using [^{35}S]methionine as the label. Translation reaction mixtures were incubated with 1.5 μg immunoaffinity purified T antigen (Stürzbecher *et al.*, 1988) and immunoprecipitated by using either normal rabbit serum (NRS), PAb419 anti-T antigen (419) or PAb421 anti-p53 (421) monoclonal antibodies. The immunoprecipitates were subjected to SDS-PAGE and autoradiography.

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CURRICULUM VITAE

Olen sündinud 29.detsembril 1957. aastal Tartus. 1980.aastal lõpetasin Tartu Riikliku Ülikooli bioloogia-geograafiateaduskonna, olles läbinud stuudiumi individuaalprogrammi järgi molekulaarbioloogia erialal. Alates ülikooliajast hakkasin uurima prokarüootse valgusünteesi molekulaarseid mehhanisme, eriti üksikute valkude funktsiooni E. coli ribosoomi peptidüültransferaasses tsentris Prof. A. Linnu ja Prof. R. Villemsi juhendamisel. 1984. aastal andis Moskva Riikliku Ülikooli kaitsmisnõukogu mulle nende uurimuste eest bioloogiakandidaadi teaduskraadi molekulaarbioloogia erialal. Peale seda huvitusin neist molekulaarsetest mehhanismidest, mis on aluseks raku normaalsele proliferatsioonile ja tumorigeneesile ning eelkõige onkogeenide ja onkovalkude uurimisest. Aastail 1985-1989 veetsin pea enamuse ajast Inglismaal Marie Curie Memoriaalfondi Instituudis Dr.John R. Jenkinsi laboris, kus keskendusin inimese onkovalgu p53 funktsiooni uurimisele. Praegu Eesti Biokeskuse teadussekretär.

I am born Dec. 29th 1957 in Tartu,Estonia. In 1980 I graduated from Tartu State University as a molecular biologist. Starting as an undergraduate student of Prof. A. Lind and Prof. R.Villems i studied molecular mechanisms of prokaryotic protein synthesis and more specifically the role of individual proteins in peptidyltransferase center of E. coli ribosome. In 1984 I got candidate of biological sciences degree from Moscow State University for these studies. After that I became interested in molecular mechanisms underlying normal cell proliferation and tumorigenesis and especially in the role of oncogenes and oncoproteins in these processes. In 1985-1989 I spent most of my time in Dr. John R. Jenkins' lab in Marie Curie Memorial Foundation Research Institute, UK. There I concentrated mostly on studies of expression and molecular mechanism of functioning of human oncoprotein p53. At the time being I am employed by Estonian Biocenter as a Secretary for Science.

The addresses:

of the author: **Estonian Biocenter**
Tähetorn, Toomemägi
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ESTONIA

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