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P53 transformation-related protein accumulates in the nucleus of transformed fibroblasts in association with the chromatin and is found in the cytoplasm of non-transformed fibroblasts

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The subcellular localization of the p53 molecule was studied in transformed and non-transformed fibroblasts. A newly established transformed cell line obtained by treating primary embryonic mouse cells *in vitro* with the chemical carcinogen methylcholanthrene was compared with the embryonic parent fibroblasts. The transformed cells lost the spindle shape characteristic of the parent fibroblasts, acquired an accelerated growth rate, developed into tumors when injected into syngeneic mice and expressed high levels of p53 synthesis estimated by immunoprecipitation of [^{35}S]methionine-labeled cell extracts. The cellular localization of the p53 molecule was studied by immunofluorescent staining of fixed cells with monoclonal antibodies and by immunoprecipitation of [^{35}S]-methionine-labeled p53 from various subcellular fractions. p53 was mainly found in the nucleus of the transformed fibroblast, while in the parent non-transformed primary embryonic cells, p53 was detected in the cytoplasm in a Triton X-100 soluble fraction, and associated with the cytoskeleton. The modulated distribution of p53 was also confirmed by analyzing a wide range of independently established transformed and non-transformed fibroblastic cell lines growing *in vitro*. The switch from the cytoplasmic localization of p53 in the non-transformed fibroblasts to a chromatin-associated accumulation in the transformed cells suggests a possible mechanism by which this protein may function in the transformed fibroblasts.

Key words: cell fractionation/immunofluorescence/p53/transformation

Introduction

p53 is a transformation-related protein encoded by the cellular genome that is synthesized at high levels in transformed cells and at low levels in non-transformed cells (DeLeo *et al.*, 1979; Jay *et al.*, 1980; Rotter *et al.*, 1980). Accentuated synthesis of the p53 molecule was observed in a wide range of cell lines (Lane and Crawford, 1979; Linzer and Levine 1979; DeLeo *et al.*, 1979; Rotter *et al.*, 1980, 1981), as well as in primary tumors in mice (Rotter, 1983). This protein is found in a variety of tissue types of several species (Kress *et al.*, 1979; Simmons *et al.*, 1980; Dippold *et al.*, 1981; Crawford *et al.*, 1981). Tumors induced by very different techniques express this protein; cells transformed by RNA or DNA tumor viruses or chemical carcinogens contain the same p53 molecule (DeLeo *et al.*, 1979; Linzer and Levine, 1979; Rotter *et al.*, 1981), suggesting that augmented synthesis of this cellular protein is probably a common secondary event following a primary signal that induces malignant transformation.

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While commonly found in numerous types of transformed cells, there are only a few experimental models of non-transformed cells where the p53 protein was detected. For example, mouse thymocytes were shown to synthesize low levels of p53, (Jay *et al.*, 1980; Rotter *et al.*, 1980), other lymphoid organs failed to manifest any p53 production which could be immunoprecipitated with specific antibodies. This molecule was also detected in primary embryonic cells but disappears as the embryonic tissue undergoes cell differentiation (Mora *et al.*, 1980; Chandrasekaran *et al.*, 1981). 3T3 fibroblasts, which are considered non-transformed cells, also synthesize low amounts of the p53, yet they were shown to contain high levels of p53-specific mRNA as assayed in a cell-free translation system (Oren *et al.*, 1981).

The major difference between p53 in transformed and non-transformed cells, as estimated by specific immunoprecipitations of [^{35}S]methionine-labeled cells, appears to be quantitative; the transformed cells produce higher levels of this protein compared with that found in non-transformed cells. Recently, however, Benchimol *et al.* (1982), have shown, using a radioimmunoassay, that the amount of p53 determined by immunoprecipitation of [^{35}S]methionine-labeled cells does not necessarily correlate with its amount as determined by the radioimmunoassay. Therefore, we decided to investigate the localization of p53 in the cell by using both immunofluorescence and cell fractionation techniques.

Earlier studies using monoclonal antibodies have shown that p53 is localized mainly in the nucleus of SV40 (Gurney *et al.*, 1980) and other transformed fibroblasts (Dippold *et al.*, 1981). However, no clear observations have been reported as to the subnuclear localization of this protein in transformed cells. Also, there were no reports on the subcellular localization of this protein in non-transformed cells. Here we report a differential localization of the p53 molecule in transformed and non-transformed cells.

Results

Increased levels of p53 in carcinogen-treated primary embryonic fibroblasts

To study the intracellular distribution of the p53 molecule in transformed *versus* non-transformed mouse fibroblasts, we established *in vitro* transformed embryonic cells and compared them with the non-treated parent cells.

Monolayers of primary embryonic cells were prepared from (BALB/c x C57BL/6)F1 embryos on the 16th day of gestation. Cells were seeded at 0.5 x 10^6 cells/plate and after 24 h several monolayer plates were treated with 5 μg/plate of methylcholanthrene dissolved in acetone as described by Berwald and Sachs (1965). Control plates were treated with acetone. The cells were incubated with the carcinogen for several days and were then split and passaged at the same cell density for several generations. At the first 10 passages, both treated and control cultures appeared similar by morphological criteria and had the same cell growth rate. After the 12th passage on one plate, the cells appeared to have a different morphology and displayed a significantly accelerated growth rate. The cell cultures were further passaged and at

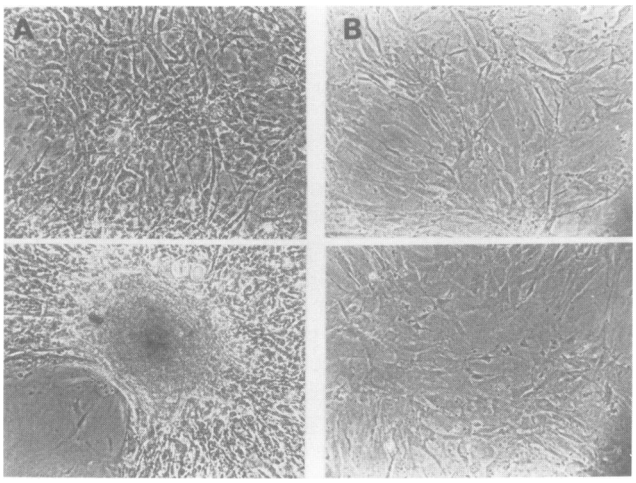

Fig. 1. Morphology of carcinogen-treated and untreated primary embryo fibroblasts. Primary embryonic fibroblasts were seeded at 0.5 x 10^6 cells/plate and treated with methylcholanthrene dissolved in acetone. The control plates were treated with acetone. The 16th passage of carcinogen-treated **(A)** and control **(B)** cells is shown.

the 16th passage they manifested a transformed phenotype very different from the untreated primary embryonic cells. The carcinogen-treated cells lost the spindle-shaped morphology and in many cases foci were evident (see Figure 1). Injection of the carcinogen-treated cells into syngeneic (BALB/c x C57BL/6)F1 mice produced local tumors after a short lag period. Based on these criteria, we refer to the carcinogen-treated cells as transformed. The carcinogen-treated fibroblasts were cloned in soft agar, single clones were picked and the following experiments were mostly performed with these cloned cells.

Normal embryonic cells and the carcinogen-treated cells were labeled with [^{35}S]methionine for 2 h and cell extracts having similar amounts of radioactive proteins were immunoprecipitated with the following sera: (a) normal serum, (b) RA3-2C2, monoclonal anti-p53 antibody, (c) PAb122 anti-p53 monoclonal antibody, (d) anti-alpha-actinin used for the detection of an abundant cytoskeletal protein. Figure 2 shows that both RA3-2C2 (lane b) and PAb122 (lane c) immunoprecipitated the p53 molecules from the extracts of carcinogen-treated primary fibroblasts (Figure 2, II). After longer exposures of the radiograms, still very little of the p53 was detected in the normal primary fibroblasts (Figure 2, I lanes b,c). The amount of [^{35}S]methionine incorporation into p53 appears to be at least 10 times higher in the carcinogen-treated cells than in the non-treated cells. In both cell types, alpha-actinin, a 110-K protein (lanes d), is present in a similar amount. This observation was repeatedly obtained with later cell passages indicating that the observed increase in the expression of the p53 molecule in the carcinogen-treated fibroblasts is not transient. The immunoprecipitated p53 molecule from the carcinogen-treated primary fibroblasts is of the same size and shares antigenic determinants with p53 found in the fibroblasts transformed by Meth A *in vivo*, shown as control in Figure 2 (M).

Subcellular localization of p53 in the carcinogen-treated embryonic cells and their non-transformed parents

The subcellular localization of the p53 molecule was studied in two ways: firstly by immunofluorescent staining of fixed cells using anti-p53 monoclonal antibodies and secondly, by immunoprecipitation of [^{35}S]methionine-labelled p53

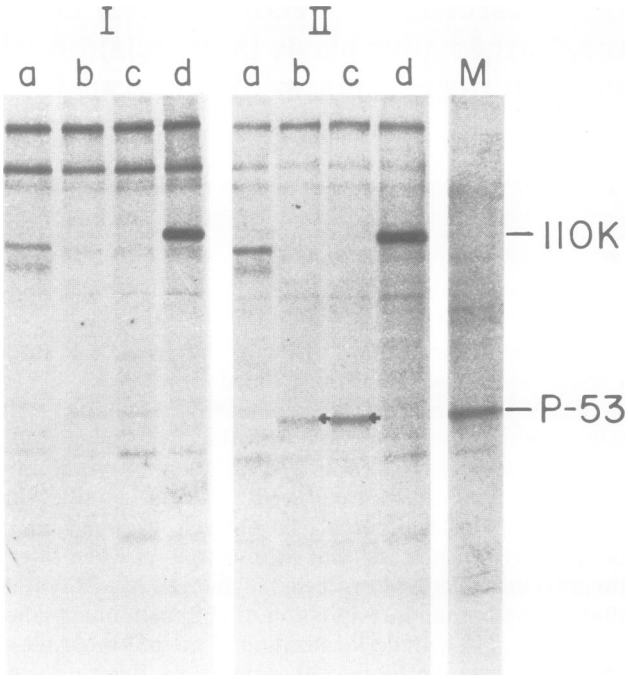

Fig. 2. Carcinogen-treated fibroblasts accumulate p53. Normal embryonic monolayers **(I)** and carcinogen **(II)** treated cells were labeled with 125 μCi/ml [^{35}S]methionine for 2 h. Cell extracts were prepared for immunoprecipitation as described in Materials and methods. Equal amounts of TCA-precipitable radioactivity were used to immunoprecipitate with: **(a)** normal serum, **(b)** RA3-2C2 monoclonal anti-p53 antibody **(c)** PAb122 anti-p53 antibody **(d)** anti-alpha-actinin serum. (M) is Meth A-transformed fibroblasts cell extracts immunoprecipitated with PAb122 anti-p53 antibody.

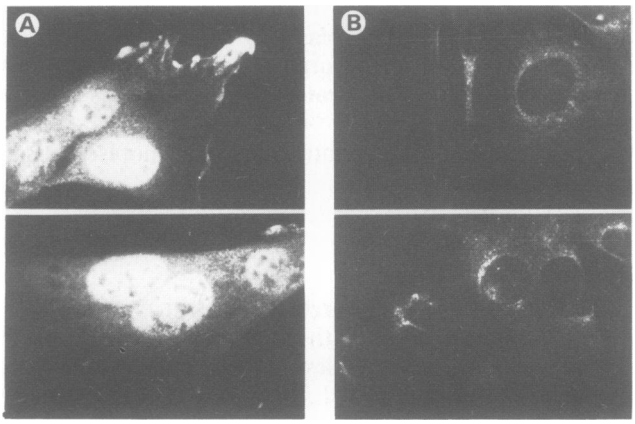

Fig. 3. Carcinogen-treated fibroblasts accumulate p53 in the cell nucleus. Primary embryo fibroblasts **(B)** and carcinogen-treated fibroblasts **(A)** were grown on glass coverslips. The cells were fixed with formaldehyde, permeabilized with Triton X-100 and processed for immunofluorescence with PAb122 anti-p53 antibody and rhodaminated goat anti-mouse antibody as detailed in Materials and methods.

molecules from various subcellular fractions with the same monoclonal antibodies.

Representative immunofluorescent staining of logarithmically growing primary embryonic fibroblasts and carcinogen-treated cells are illustrated in Figure 3B and A, respectively. By using the RA3-2C2 or PAb122 as first antibodies followed by rhodaminated anti-rat or anti-mouse immunoglobulin, respectively, we obtained mainly nuclear staining in the carcinogen-treated primary fibroblasts (Figure

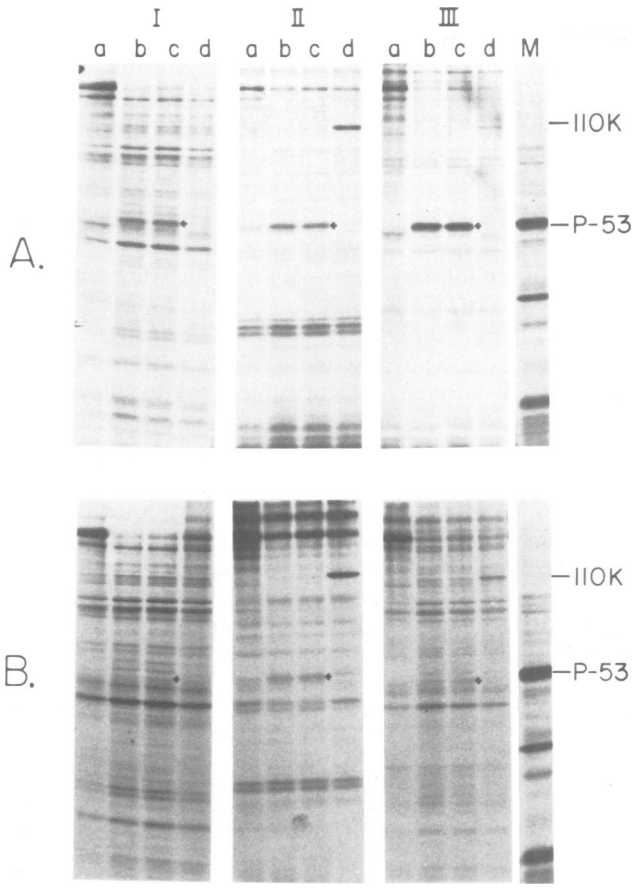

Fig. 4. Distribution of p53 in subcellular fractions of carcinogen-treated and untreated fibroblasts. Carcinogen-treated **(A)** and untreated primary fibroblasts **(B)** were labeled for 2 h with 125 μCi/ml [^{35}S]methionine. The cells were gently lysed on the plate with Triton X-100 and the Triton-soluble fraction was removed **(I)**. The cells were scraped into the DOC/Tween 40 mixture (Penman, 1966) to remove the cytoskeletal material from the nuclei and the nuclei **(III)** were sedimented. The supernatant was considered the cytoskeletal fraction **(II)**. From each subcellular fraction equal amounts of radioactive material were immunoprecipitated with: **(a)** normal serum, **(b)** RA3-2C2, **(c)** PAb122, **(d)** anti-alpha-actinin serum. M – control for p53 from Meth A-transformed fibroblasts (see Figure 2).

3A), while the non-treated, parental cells manifest a fine granular staining of the cytoplasm (Figure 3B). When 500 cells of each type were counted at random no specific nuclear staining was observed in the non-transformed cells and all cells of the carcinogen-treated fibroblasts displayed the strong nuclear fluorescence shown in Figure 3A. These results, showing modulation in the localization of the p53, were also confirmed by following the presence of this molecule in the various subcellular fractions. Carcinogen-treated and untreated primary embryonic cells were labelled for 2 h with [^{35}S]methionine. Each cell line was fractionated into three major fractions. We have employed a fractionation scheme that separates the soluble from the structural components of the cytoplasm (Ben Ze'ev *et al.*, 1979, 1981; Brown *et al.*, 1976; Webster *et al.*, 1978). A cytoplasmic soluble fraction was obtained by treating the monolayer culture with 0.5% Triton X-100 (I) and removing the supernatant. This treatment leaves intact the cytoskeletal components that hold the nucleus (Ben Ze'ev *et al.*, 1979). To solubilize the cytoskeletal fraction, and separate it from the nuclei, the Triton X-100 extracted cell layer was scraped into a mixture of 0.5% DOC and 1% Tween 40. This method effectively removes cytoplasmic components from the nucleus as assayed by electron microscopy but does not remove DNA, nuclear RNA and histones from nuclei (our unpublished results, Holzman *et al.*, 1966; Ben-Ze'ev *et al.*, 1981, 1982; Capco *et al.*, 1982). After vortexing, the nuclei were sedimented (III) and the supernatant containing the solubilized cytoskeletal fraction (II) was saved.

Equivalent amounts of these subcellular fractions obtained from both cell lines were immunoprecipitated with the following antibodies: (a) normal serum, (b) RA3-2C2, (c) PAb122, (d) anti-alpha-actinin. The results presented in Figure 4 clearly show that p53 molecules of the carcinogen-treated cells, evaluated by immunoprecipitation with the two anti-p53 monoclonal antibodies (lanes b and c), are concentrated mainly in the nuclear fraction, (Figure 4A, III) and much lower amounts are also found in the Triton-soluble fraction (Figure 4A, I) and in association with the cytoskeletal framework (Figure 4A, II). These autoradiograms were overexposed to detect the low levels of p53 in the various fractions. Therefore, the amount of nuclear p53 seen in Figure 4A III is no longer in the linear range of intensity and it is probably an underestimate of the real value. The parental embryonic fibroblasts (Figure 4B) have a low level of p53 synthesis and these p53 molecules are not associated with the nuclear fraction, (Figure 4B, III) but rather are found in the subcytoplasmic fractions mainly associated with the cytoskeleton (see Figure 4B, II, lanes b,c). The alpha-actinin molecules are mainly found in the cytoskeletal fraction, as expected, in both the carcinogen-treated and non-treated cells (Figure 4A, II, 4B, II lanes d).

Localization of p53 in transformed and non-transformed established cell lines

Since these observations might be unique to the primary embryonic fibroblast system, it was important to study a wide range of established transformed and non-transformed fibroblastic cell lines.

The following transformed cell lines were studied: Meth A, a chemical carcinogen-induced fibrosarcoma; B-16 F1, a melanoma; UV 2237-P, a u.v. radiation-induced fibrosarcoma; SV-101, an SV40-transformed Swiss 3T3 cell line; and ANN-1, an Abelson-transformed Swiss 3T3 cell line. The non-transformed cell lines studied included the 3T3 Swiss cells, the A31 BALB/c 3T3 cells and fibroblastic embryonic cells that were in culture for <20 passages. Localization of the p53 molecule in these cell lines was evaluated by both immunofluorescent staining with the monoclonal anti-p53 antibodies as well as by immunoprecipitation of [^{35}S]-methionine-labeled p53 from the various subcellular fractions.

Figure 5 presents some of the results obtained when the immunofluorescent staining was performed using the PAb122 anti-p53 antibodies. RA3-2C2 anti-p53 monoclonal antibodies yield the same staining profile. It is clear from Figure 5 that p53 molecules accumulate in the nuclei of the transformed cells tested here. SV-101 (d) UV-2237-P (e), B16 melanoma (f) and Meth A (g) cells, with some variations, have a clear nuclear staining, as well as a mild cytoplasmic staining. The Meth A-transformed cells spread very poorly and often form multinucleated giant cells, but even in these the fluorescence is clearly nuclear (Figure 5g). The staining of these transformed cells closely resembles that seen in the carcinogen-treated primary fibroblasts as shown in Figure 3A. On the other hand, the immunofluorescent staining of

the 3T3 Swiss fibroblasts (Figure 5b), manifests a fine granular staining of the cytoplasm and the perinuclear area without preferential staining of the nucleus. The cytoplasmic staining of the non-transformed fibroblasts represents mainly cytoskeleton-associated p53, since it is not removed by treating the cells with Triton X-100 (data not shown). A similar immunofluorescent staining pattern was also obtained in the long-term primary embryonic cultures (Figure 5a) and in the A31 BALB/c 3T3 cells that tend to become transformed spontaneously and produce large amounts of p53 (Ben-Ze'ev *et al.*, in preparation) (Figure 5c). This differential distribution of p53 in the transformed *versus* the non-transformed cells was also confirmed when analyzing these cell lines by the cell fractionation method described above. Figure 6 illustrates an example of the distribution of the p53 molecule in the Meth A-transformed fibroblastic cell lines studied by cell fractionation. [^{35}S]methionine-labeled Meth A cells were fractionated and equivalent amounts of each fraction were immunoprecipitated with the following antibodies: (a) normal serum, (b) RA3-2C2, (c) PAb122, (d) goat anti-Moloney. Figure 6 shows that the p53 molecules are detected mainly in the nuclear fraction (Figure 6 III) and minor amounts of this protein and a possible breakdown product of ~45 K is found in the soluble fraction and cytoskeletal fraction (Figure 6 I, II respectively, lanes b and c). In pulse-chase experiments followed by the fractionation scheme described in Figure 6, we found that it takes 10–15 min for the newly synthesized p53 to reach its nuclear location. Therefore, in the 2 h labeling protocols used in the experiments summarized in Figures 4 and 6, we were probably determining the final location of p53 rather than a transient location. Figure 6, group IV lanes b and c represent the p53 found in total cell extracts lysed in phospholysis buffer immunoprecipitated

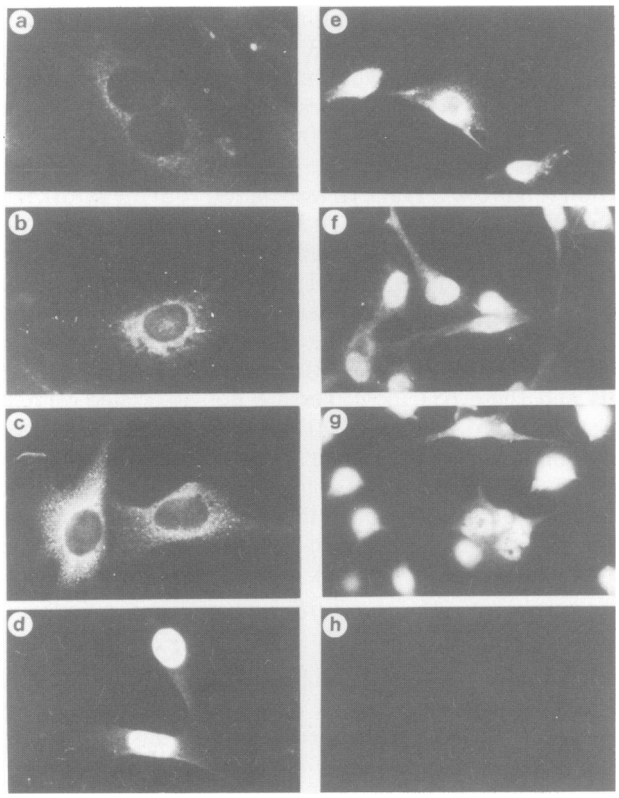

Fig. 5. Localization of p53 in transformed and non-transformed established cell lines. Cells were grown on glass-coverslips and either fixed **(a,b,c,d,g,h)** and permeabilized as described in Figure 3 or first extracted **(e,f)** with Triton X-100 to prepare Triton cytoskeletons prior to fixation with formaldehyde. The cells were processed for immunofluorescence with PAb122 anti-p53 antibody as described in Figure 3. **(a)** Long-term primary mouse fibroblasts, **(b)** Swiss 3T3, **(c)** BALB/c 3T3 (A31), **(d)** SV-101, **(e)** UV-2237 P fibrosarcoma, **(f)** B16-F1 melanoma, **(g)** Meth-A, **(h)** SV101 with non-immune serum.

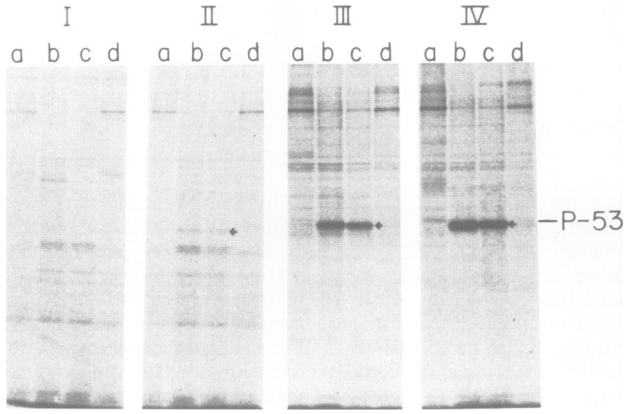

Fig. 6. Distribution of p53 in the subcellular fractions of a transformed established cell line. Meth A-transformed cells were labeled with [^{35}S]-methionine as described in Figure 2 and fractionated into Triton X-100 soluble **(I)**, cytoskeletal fraction **(II)**, and a nuclear fraction **(III)** as described in Figure 4. Control of total cell lysate was also included **(IV)**. Equal amounts of radioactive proteins were immunoprecipitated with **(a)** normal serum, **(b)** RAC-2C2, **(c)** PAb122, **(d)** goat anti-Moloney serum.

Table I. Subcellular localization of p53 molecules in transformed and non-transformed fibroblasts

Fibroblastic cell lines	Method of transformation	Genetic origin of mouse	Subcellular localization of p53[a]	
			Cytoplasmic	Nuclear
Primary embryonic fibroblasts:				
passage 12	–	(C57BL/6 x BALB/c)F_1	+	–
passage 21	–	(C57BL/6 x BALB/c)F_1	+	–
3T3	–	NIH Swiss	+	–
A31	–	BALB/c	+	–
Carcinogen-treated embryonic fibroblasts	*In vitro* treatment with methylcholanthrene	(C57BL/6 x BALB/c)F_1	±	+ + +
ANN-1	Abelson murine leukemia virus	NIH Swiss	±	+ + +
SV-101	SV40 virus	NIH Swiss	±	+ + +
Meth A	*In vivo* treatment with methylcholanthrene	BALB/c	±	+ + +
B16-F1	Spontaneous melanoma	C57/BL/6	±	+ + +
UV-2237-P	u.v.-induced fibrosarcoma	C3H/eb	±	+ + +

[a]p53 was estimated by both immunoprecipitation of [^{35}S]methionine-labeled proteins with specific monoclonal antibodies from the various cell fractions and by immunofluorescent staining with the same monoclonal antibodies.

with RA3-2C2 and PAb122. In the 3T3 Swiss cells, as in the embryonic primary fibroblasts, the p53 molecules which can be observed after very long exposures of the radiograms were detected mainly in the two subcytoplasmic fractions (data not shown). Table I summarizes the subcellular distribution of the p53 molecules in the various cell lines studied.

Subnuclear localization of p53 in transformed fibroblasts

To localize the subnuclear distribution of p53, nuclei were treated with DNase and salt to remove ~95% of the DNA and histones. This fractionation yields a solubilized chromatin fraction and leaves behind the skeletal framework of the nucleus, the nuclear matrix (Berezney and Coffey, 1974; Ben-Ze'ev *et al.*, 1982; Capco *et al.*, 1982). We compared the relative amount of p53 in the solubilized chromatin fraction and in the nuclear matrix fraction in SV40-transformed and methylcholanthrene-transformed fibroblasts by the immunofluorescence and immunoprecipitation procedures as used above. A nuclear preparation was divided into two: one half was used for immunoprecipitation and from the other half the chromatin fraction and the nuclear matrix fraction were prepared before immunoprecipitating with monoclonal antibodies against p53. Figure 7 shows that in both SV40-transformed (Figure 7B) and methylcholanthrene-transformed (Figure 7A) cell lines, p53 is removed from the nuclei together with the chromatin (group II) as assayed by immunoprecipitation. The nuclear fluorescence in Triton cytoskeletons of SV40-transformed cells stained with PAb122 and rhodaminated goat anti-mouse IgG (Figure 7C), is by and large removed in preparations in which the Triton cytoskeletons were treated with DNase and salt to solubilize the chromatin (Figure 7D).

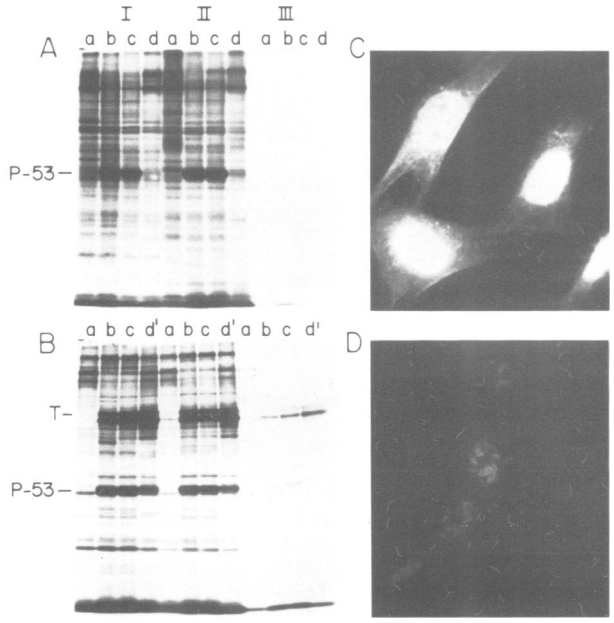

Fig. 7. Association of p53 with the chromatin fraction in SV40- and methylcholanthrene-transformed cells. Meth A **(A)** and SV-101 **(B)** were labeled with [^{35}S]methionine for 2 h and nuclei prepared with Triton X-100 and with DOC/Tween as described in Figure 6. One half of the samples were treated with DNase and salt to extract the DNA and histones **(II)** and to obtain nuclear matrices **(III)** as described in Materials and methods. The other half of the samples **(I)** and fractions II and III were used for immunoprecipitation with **(a)** normal serum, **(b)** RA3-2C2, **(c)** PAb122, **(d)** goat anti-Moloney serum, **(d')** hamster anti-T serum. For indirect immunofluorescence studies, SV-101 cells grown on coverslips were stained with PAb122 and rhodaminated anti-mouse IgG as described in Figure 3. **(C)** Triton cytoskeletons, **(D)** nuclear matrices obtained as in fraction III.

Discussion

p53, a protein encoded by the cellular genome, was shown to be synthesized at elevated levels in transformed cells. The function of this protein is not clear, several investigators have, however, suggested that it plays a role in cell division (Milner and Milner, 1981; Campisi *et al.*, 1982; Mercer *et al.*, 1982). The mechanism(s) controlling the expression of this protein in the transformed cell are also unknown. In certain experimental systems it was suggested that binding of this protein to viral-encoded proteins, such as the large T antigen of SV40 leads to the stabilization of p53, which otherwise has a short lifespan (Linzer and Levine, 1979; Oren *et al.*, 1981).

Previous studies have suggested that the major difference between the p53 of transformed and non-transformed cells is quantitative. Transformed cells synthesize significantly higher levels of this protein compared with non-transformed cells (Jay *et al.*, 1980; Rotter *et al.*, 1980). Non-transformed cells in which this protein is synthesized at low concentrations detectable by specific immunoprecipitation are normal mouse thymocytes, (Jay *et al.*, 1980; Rotter *et al.*, 1980), primary embryonic fibroblasts (Mora *et al.*, 1980; Chandrasekaran *et al.*, 1981) and 3T3 fibroblast cell lines growing *in vitro* (Oren *et al.*, 1981). In the present study we used cell fractionation techniques to study both the biosynthetically labeled p53 molecules and the steady-state distribution of this protein estimated by immunofluorescent staining. Since we have observed similar results using two independently established anti-p53 monoclonal antibodies, it is likely that the product followed by immunofluorescent staining is identical to that observed by the immunoprecipitation method. Our results show that, in addition to the quantitative difference in p53 synthesis previously observed, the p53 molecule seems to be located in different subcellular compartments in the transformed and non-transformed fibroblasts. Immunofluorescent staining, as well as specific immunoprecipitation of metabolically labelled p53 molecules from various cell fractions, showed that this protein is mainly concentrated in the nuclei of transformed cells and only a minor fraction was detected in the cytoplasmic compartments. In the non-transformed cells, however, p53 is detected in the cytoplasmic compartment only. The p53 molecules of the transformed and non-transformed cells are indistingishable in their size and antigenic determinants, as evaluated by binding to two types of monoclonal anti-p53 antibodies. The differential subcellular distribution of p53 molecules we detect in transformed and non-transformed cells appears to be restricted to the fibroblastic cell population only. Previously we detected p53 in the cytosol of lymphoid cells transformed by the Abelson murine leukemia virus (Rotter *et al.*, 1981), in the present study we observed that the great majority of p53 is easily released from these cells by even mild treatment with Triton X-100.

The accumulation of p53 in the nuclei of transformed fibroblasts may suggest a possible mechanism for the stabilization of this protein by direct binding to nuclear elements such as the chromatin. When the chromatin is solubilized by digestion with DNase and high salt, leaving behind a nuclear matrix (Berezney and Coffey, 1974; Ben-Ze'ev *et al.*, 1982), p53 is removed together with the chromatin. Treatment with DNase alone which does not

remove the chromatin from nuclei, also does not remove p53. In some cases, such as cells transformed by SV40, it has been suggested that the nuclear localization of the p53 molecule is mediated by its binding to the large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979; Gurney, 1981). We found that in carcinogen-treated primary fibroblasts and in the other established non-SV40 transformed cell lines tested here, p53 accumulates in the nucleus, but it was not detected in a complex with a specific protein. We suggest, therefore, that the migration of p53 into the nuclei of transformed cells may involve a procedure leading to the stabilization and subsequent accumulation of this protein. It is not clear, however, whether the accumulation of p53 in the nucleus is the result or the cause of cellular transformation.

Finally, the differential subcellular localization of p53 in transformed *versus* non-transformed fibroblasts, might provide a cytological diagnostic method for the detection of malignant transformed cells.

Materials and methods

Cell lines

The following cell lines were used: Meth A, an established cell line induced by methylcholanthrene treatment of BALB/c mice (DeLeo *et al.*, 1977). ANN-1, a 3T3 Swiss mouse fibroblast transformed by the Abelson murine leukemia virus, (Scher and Siegler, 1975) SV-101, a subclone of 3T3 Swiss mouse fibroblast transformed by SV40 was obtained from R.Pollack (Columbia, NY). The B16-F1 line was derived from pulmonary metastases produced by i.v. injection of B16 melanoma cells (Fidler, 1973) and the UV-2237-P which is the parental fibrosarcoma induced in a female C3H mouse by chronic u.v. radiation (Kripke *et al.*, 1978) were obtained from A.Raz (The Weizmann Institute, Rehovot). 3T3 Swiss mouse fibroblasts were obtained from H.Green (MIT, Cambridge, MA). A31 is a 3T3 mouse fibroblast derived from BALB/c. Primary mouse cells were prepared from (BALB/c x C57BL/6)F1 embryos at 16–18 days of gestation. The monolayers were prepared from mildly trypsinized cells cultured *in vitro*. All the above fibroblastic cells were grown in RPMI-1640 or Dulbecco's modified Eagle medium with 10% heat-inactivated fetal calf serum. For the immunofluorescent staining, cells were grown on sterile 18 mm glass coverslips and were used within 24–48 h after seeding. The hybridoma cell lines RA3-2C2 (Rotter *et al.*, 1980; Coffman and Weissman, 1981) and the PAb122 (Gurney *et al.*, 1980) were grown in RPMI-1640 medium enriched with 20% heat-inactivated fetal calf serum supplemented with 20 mM L-glutamine and 20 mM Na-pyruvate.,

Antibodies

Anti-p53 monoclonal antibodies were obtained from the established cell lines RA3-2C2 and PAb122. Supernatants of these cell lines were collected from confluent cultures and protein was concentrated by ammonium sulfate precipitation. Goat anti-Moloney virus protein antibodies were obtained from the Division of Cancer Cause and Prevention National Center Institute, NIH, Bethesda, MD. Anti-alpha-actinin was a generous gift from B.Geiger of the Weizmann Institute of Science, Rehovot. Normal serum was collected from 2 month-old female BALB/c mice.

Cell labeling and immunoprecipitation

Radioactive chemicals were purchased from Amersham, UK. Cell monolayers were washed several times with phosphate buffered saline (PBS) and then covered with 2 ml of Dulbecco's modified Eagle medium without methionine enriched with 10% dialyzed heat-inactivated fetal calf serum and 250 μCi of [^{35}S]methionine. Cell monolayers were incubated for 2 h, the excess radioactive material was removed and cells were washed several times with PBS. Cell monolayers were either fractionated as described below, or immediately extracted into 5 ml lysis buffer (10 mM NaH_2PO_4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS).

Fractions of 0.5–1 ml cell lysate (an equal amount of radioactive labeled proteins) were immunoprecipitated with control or specific antibodies. Antigen-antibody complexes were collected by binding to *Staphylococcus aureus* (Kessler, 1975). SDS-polyacrylamide gel electrophoresis was performed on the discontinuous stacking system of Laemmli (1970).

Immunofluorescence

The cells were grown on glass coverslips and prepared for immunofluorescence by fixation with 3.5% formaldehyde for 30 min at room temperature and permeabilization with 1% Triton X-100 in PBS for 10 min. Alternatively, the cells were first extracted with Triton X-100 in extraction buffer (see below) to prepare 'Triton cytoskeletons' which were then fixed with 3.5% formaldehyde. The PBS-washed coverslips were incubated with undiluted monoclonal antibody to p53 or with non-immune mouse antiserum, for 30 min at room temperature. The coverslips were washed with PBS and incubated with rhodaminated goat anti-mouse or rat antibody (3 fluorophores/molecule) a gift from B.Geiger, at 5–10 μg/ml. After 30 min incubation, the coverslips were washed extensively with PBS and mounted in Gelvatol and viewed in a Zeiss photomicroscope III.

Cell fractionation

The fractionation into the Triton X-100 soluble, cytoskeletal and nuclear fraction was similar to the procedure described by Ben-Ze'ev *et al.* (1979, 1981). Briefly, the cells were washed twice with cold PBS and once with extraction buffer (50 mM NaCl, 10 mM Hepes, pH 7.4, 2.5 mM $MgCl_2$, 300 mM sucrose and 1 mM PMSF). Extraction was achieved by adding the above buffer with 0.5 to 1% Triton X-100 to the washed cells, for 5 min on ice. The Triton X-100 soluble fraction was removed. The cytoskeletons were solubilized by scraping them into DOC/Tween buffer (Penman, 1966), containing 10 mM NaCl, 10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 0.5% deoxycholate, 1% Tween 40 and 1 mM phenylmethylsulphonyl fluoride, followed by homogenization by pipetting. The nuclei were pelleted at 2000 r.p.m. for 2 min at 4°C and the solubilized cytoskeletons removed. The nuclei were further fractionated in some experiments by a treatment with 1 mg/ml RNase-free DNase for 30 min at 4°C followed by the slow addition of NaCl to a final concentration of 1 M. After centrifugation at 1000 r.p.m. for 5 min at 4°C the supernatant containing the DNA and histones was removed and the pellet containing the nuclear matrix was dissolved in lysis buffer. The various fractions were brought to 1x lysis buffer (see above) and processed for immunoprecipitation.

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