p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells

(immunoprecipitation/peptide map/phosphoprotein/tumor antigen)

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ABSTRACT p53, a transformation-related cellular-encoded protein, was found to accumulate at high concentration in transformed cell lines. The results presented here show that p53 biosynthesis is also increased in most induced and spontaneous mouse tumors. Judged by the identity in antigenic determinants (estimated by binding to monoclonal antibodies), size, and partial peptide mapping, I conclude that the p53 molecule found in primary tumors is indistinguishable from that in established cell lines. The fact that p53 is found in heterogeneous populations of primary tumors makes it a convenient biochemical diagnostic marker for the detection of primary tumors in mice. It is found in primary tumors as a phosphoprotein, just as it was found previously in established cell lines. On the other hand, the p53 found at low concentration in normal thymocytes is labeled with [^{35}S]methionine but cannot be found in its phosphorylated form.

There are several reasons to assume that p53, a cellular-encoded gene product, is a transformation-related protein. First, p53 accumulates at high concentrations in malignant transformed cells, reaching levels 50- to 100-fold those found in normal thymocytes (1, 2). The protein is described as a phosphoprotein, 50–55 kilodaltons in size, commonly found in a variety of transformed cells, with no restriction as to the manner of tumor induction, the tissue type, or the species specificity (3–8), suggesting that it is encoded by a well-conserved gene. Second, in some cases, p53 has been found complexed with virally encoded tumor antigens. In cells transformed by the simian virus 40 DNA tumor virus, nonviral middle tumor antigen, p53 was found to be complexed with the virally encoded large tumor antigen (4, 5, 9, 10). This complex was detected mainly in the nuclei of the transformed cells (11). Moreover, in Epstein–Barr virus-transformed cells, a p53 host-encoded product was found to copurify with the virally encoded antigen (12, 13). In many other cases, however, the p53 molecule was detected as a single product, uncomplexed to any other known proteins. For example, the p53 of Abelson murine leukemia virus (A-MuLV)-transformed lymphocytes is detected (by immunoprecipitation) as a single molecule, similar to that found in chemically transformed Meth A fibroblasts (8). The third argument concerns the fact that p53 is a phosphoprotein found to be phosphorylated on a serine residue (8, 14). p53 can be a putative substrate product for the "onc" phosphoproteins occurring concomitantly in various transformed cells. Fourth, although this protein is a cellular-encoded product and can be defined in immunological terms such as a "self" protein, there are occasions when p53 induces the production of specific antibodies against itself. In an earlier study, Rotter et al. (1) observed that mice bearing a syngeneic Abelson-induced tumor produced anti-p53

antibodies at a late stage of tumor development. These mice also produced anti-P120, the viral *onc* gene encoded by the A-MuLV genome (15) at a frequency similar to that of the anti-p53 antibodies (1). This implies that accumulation of the p53 product in transformed cells leads to breakdown of the immunological tolerance system controlling the p53 antigen in normal cells. Recently, several monoclonal antibody cell lines that specifically immunoprecipitate the p53 molecule have been established, thus facilitating the detection of this protein (1, 7, 11, 16).

All these observations suggest therefore that the cellular-encoded protein p53 can be used as a marker for established transformed cells. In earlier experimental models, the p53 molecule was studied in *in vitro* established cell lines, in particular those induced by viruses and chemical carcinogens (1–14, 16). It was thus important to test whether the protein is found at high concentration in primary mouse tumors, rather than being only a tissue culture artefact. In the present experiment, I therefore studied the expression of p53 in primary induced or spontaneous tumors in mice, with the idea that detection of elevated cellular p53 concentrations could serve as a biochemical diagnostic tool for primary malignant tumors.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c female and male mice 4–12 wk old were obtained from the Weizmann Institute Animal Breeding Center.

Cell Lines. 2M3 is a nonproducer A-MuLV-transformed lymphoid cell line. 2M3/M is derived from 2M3 but contains the Moloney helper virus. The lines were grown in RPMI 1640 medium/10% heat-inactivated fetal calf serum (BioLab, Israel)/20 mM 2-mercaptoethanol. The hybridoma cell lines RA3-2C2 (1, 17) and PAb122 (11) were grown in RPMI 1640 medium/20% heat-inactivated fetal calf serum/20 mM L-glutamine/20 mM Na pyruvate.

Mouse Tumors. The tumors studied were donated by N. Haran-Ghera of the Department of Chemical Immunology of this Institute. The tumors were solid and developed in the thymus or lymphatic modes or arose spontaneously in other sites. Tumor cell suspensions were prepared from the primary induced or spontaneous tumors. Local tumors were excised from the organs, necrotic areas were removed, and a cell suspension was prepared by mashing the tumor mass through a stainless steel mesh. The single-cell suspensions were washed several times in phosphate-buffered saline at room temperature and the number of live cells was determined by trypan blue exclusion.

Abbreviations: A-MuLV, Abelson murine leukemia virus; M-MuLV, Moloney murine leukemia virus; MNUA, methylnitrosourea amine; i.v., intravenous(ly); RadLV, radiation leukemia virus.

Normal Spleen and Thymus Cell Suspensions. Spleens or thymuses were removed from normal mice, cell suspensions were prepared, and cells were counted as described above.

Immune Sera. Anti-p53 antibodies were obtained from the following sources. (i) Tumor-bearing (Tb) serum, a polyclonal serum, was collected from BALB/c mice bearing A-MuLV-induced 2M3/M tumors (1). (ii) Monoclonal antibodies were obtained from the established cell lines RA3-2C2 (1, 17) and PAb122 (11) and concentrated by ammonium sulfate precipitation. Goat anti-Moloney murine leukemia virus (M-MuLV) antibodies were obtained from the Division of Cancer Cause and Prevention of the National Cancer Institute.

Cell Labeling and Immunoprecipitation. Radioactive chemicals were purchased from Amersham, England. Cells were washed several times with phosphate-buffered saline and then suspended in labeling medium at a concentration of 10^7/ml. For [^{35}S]methionine labeling, cells were suspended in 1.5 ml of methionine-lacking Dulbecco's modified Eagle's medium/ 10% dialyzed heat-inactivated fetal calf serum containing 125 μCi of [^{35}S]methionine (1 Ci = 37 GBq). Cells were incubated for 2 hr and washed in phosphate-buffered saline, the pellet was extracted with 5 ml of lysis buffer (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.5/100 mM NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1% NaDodSO$_4$) at 0°C, and the suspension was clarified at 150,000 × g for 1 to 2 hr. For ^{32}P labeling, cells were suspended in 2 ml of PO$_4$-lacking Dulbecco's modified Eagle's medium/10% dialyzed heat-inactivated fetal calf serum containing 500 μCi of ^{32}P. Cells were incubated for 4–6 hr and extracts were prepared as described above. Some of the tumors were highly contaminated with normal B cells and therefore they were repeatedly treated with *Staphylococcus aureus* to remove the immunoglobulin background that comigrated with the p53 product.

Fractions of cell lysate (0.5–1 ml) (determined by the amount of labeled protein) were immunoprecipitated with control or specific antibodies. Antigen–antibody complexes were collected by binding to *S. aureus* (18). NaDodSO$_4$/polyacrylamide gel electrophoresis was performed on the discontinuous stacking system of Laemmli (19).

Partial Protease Digestion. Proteolytic digestion was carried out essentially as described by Cleveland (20). The specific immunoprecipitation product was separated by electrophoresis through NaDodSO$_4$/polyacrylamide gels, and the bands of interest were cut out from the wet gel. The fragments were washed several times in 0.1% NaDodSO$_4$/1 mM EDTA/0.125 mM Tris·HCl, pH 6.8, and then applied to a second gel. *S. aureus* V8 protease (Miles) was added at several concentrations to individual fragments, and the products were concentrated at the interphase between the stacking and running gels and left to stand at room temperature for 30 min before resuming electrophoresis.

RESULTS

The cellular-encoded protein p53 is abundant in established cell lines of various tissue and cell specificities grown *in vitro* (1–4).

In the present experiments, I tested primary mouse tumors representing a heterogeneous group with regard to tissue specificity, genetic origin, and manner of tumor induction. The first group tested were individual tumors characterized by their cell-surface marker as T-cell lymphomas. These tumors were induced in 2-month-old C57BL/6 mice by 1 mg of methylnitrosourea amine (MNUA) by either intravenous (i.v.) injection or carcinogen feeding. Most of these mice developed thymomas within 80–120 days. The second group included thymecto-

mized C57BL/6 or AKR mice in which primary tumors were induced by i.v. injection of MNUA. In this group, tumors developed in the spleen or peripheral lymphoid organs and were characterized as null lymphomas. The third group consisted of lymphomas induced in C57BL/6 mice by a combination of injection of D-radiation leukemia virus (RadLV) followed by 400 rads of x-irradiation (1 rad = 0.01 gray) or by injection of A-RadLV. These tumors developed in the thymus and were characterized as T-cell lymphomas. The fourth group consisted of two primary mammary adenocarcinomas that arose spontaneously in AKR and BALB/c mice. The fifth group consisted of a B-cell lymphoma that arose spontaneously in a C3H/eb mouse, and the sixth group consisted of spontaneous tumors in aged AKR mice. [^{35}S]Methionine-labeled cell lysates were prepared from the primary tumor cells and occasionally from a first passage in a syngeneic mouse. Cell lysates were immunoprecipitated with the following anti-p53 antibodies: (i) Tb serum, a polyclonal antibody; (ii) RA3-2C2, (iii) PAb122; the two latter are specific anti-p53 monoclonal antibodies. The RA3-2C2 that immunoprecipitated the p53 from an extract of cells labeled with [^{35}S]methionine for 2 hr also shares some antigenic determinants with a membranal 35-kilodalton protein (unpublished data). The level of p53 observed as a single band on NaDodSO$_4$/polyacrylamide gels was compared with that found in the 2M3/M established A-MuLV-transformed cell line tested in the same experiment.

The results obtained are summarized in Table 1. The primary tumors tested contained the p53 molecule that immunoprecipitated with Tb serum, RA3-2C2, and PAb122 to the same extent. The amount of p53 synthesized in these tumors varied slightly; whether this reflects a variation in the cellular content of this molecule or whether it is a result of contamination with variable numbers of nontumor cells occurring in the tumor tissue is unclear.

In these experiments, a constant number of tumor cells was labeled and the same amount of labeled protein was then immunoprecipitated. The 2M3/M line was incubated as a control and run on each gel as a reference.

The spontaneous thymomas of AKR mice were the only group of primary tumors in which no p53 could be found after immunoprecipitation with RA3-2C2, PAb122, and Tb serum. The lack of expression of p53 in this tumor could be the result of a technical problem (e.g., the reagents may have failed to detect this protein in AKR mice because the product is truncated). But it may be an authentic observation, reflecting the fact that, for certain reasons, these tumors lack the expression of the protein.

Some of the results summarized in Table 1 are shown in Fig. 1. The spontaneous mammary adenocarcinoma contained both the p53 molecule (lanes b and c) and viral particles that cross-hybridized with goat anti-M-MuLV serum (lanes d). Although these two spontaneous tumors (MT-147-800 and MT-6) were isolated from individual mice differing in their genetic origins, they seem to have similar profiles for proteins that immunoprecipitate with goat anti-M-MuLV serum, suggesting that these products are specific for the mammary virus.

When chemically induced tumors (e.g., 148-54, 148-153) were analyzed, I detected the p53 product by immunoprecipitation with anti-p53 monoclonal antibodies; no specific product was observed when these cell lysates were immunoprecipitated with goat anti-M-MuLV serum. This suggests that these tumors do not involve the induction of viral particle expression that is detected with the polyclonal goat anti-M-MuLV serum.

The p53 found in the above-described primary tumors shares antigenic determinants with the p53 of established lines. This is evidenced by the binding of the protein with the same mono-

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Table 1. p53 transformation-related protein in primary mouse tumors

	Mouse strain	Tumor type	Method of induction	Latency, days	p53
2M3 cell line	BALB/c	Pre-B leukemia	A-MuLV		100
Tumor*					
148-54	C57BL/6	T-cell lymphoma	MNUA† (i.v.)	110	106
148-153	C57BL/6	T-cell lymphoma	MNUA (i.v.)	110	98
148-171	C57BL/6	T-cell lymphoma	MNUA (feed)	95	286
148-172	C57BL/6	T-cell lymphoma	MNUA (feed)	104	94
148-181	C57BL/6	T-cell lymphoma	MNUA (feed)	98	112
148-192	C57BL/6	T-cell lymphoma	MNUA (feed)	118	118
148-193	C57BL/6	T-cell lymphoma	MNUA (feed)	119	121
148-50	C57BL/6‡	Null lymphoma	MNUA (i.v.)	76	97
147-708	AKR‡	Null lymphoma	MNUA (i.v.)	110	115
68-127-70	C57BL/6	T-cell lymphoma	D-RadLV (400 rads of x-irradiation)	90	136
59-127-4	C57BL/6	T-cell lymphoma	D-RadLV (400 rads of x-irradiation)	80	124
50-136-91	C57BL/6	T-cell lymphoma	A-RadLV	95	93
147-800	AKR	Mammary adenocarcinoma	Spontaneous	250	112
MT-6	BALB/c	Mammary adenocarcinoma	Spontaneous	150	83
38C-14	C3H/eb	B-cell lymphoma	Spontaneous	150	312
1-5	AKR	T-cell lymphoma	Spontaneous	93	3

p53 was determined by immunoprecipitation of a constant amount of radioactive trichloroacetic acid-precipitable material from the [^{32}S]methionine-labeled tumor cell extract with monoclonal anti-p53 antibodies (RA3-2C2 and PAb122) and with polyclonal Tb serum. In each individual experiment, both the intensity of the radiogram and the cpm of the p53 band were compared with those of the 2M3 cell lysate. p53 in each individual tumor is expressed as percentage of that found in 2M3 cell line.
* Donated by N. Haran-Ghera.
† Two-month-old animals were treated with 1 mg of MNUA either by i.v. injection of a saline solution or oral administration of a polyethylene glycol 400 solution.
‡ Animals thymectomized at age 1 month.

clonal antibodies. In the experiment described below, I compared the partial digestion products of the p53 molecules from primary tumors with those from the established cell lines.

The partial digestion products obtained when the p53 from various tumors (148-181, 148-193, T-cell thymomas, 148-50, null lymphomas, MT-6 mammary adenocarcinomas of AKR origin and MT-140-800 mammary adenocarcinomas of BALB/c origin, and the 2M3 A-MuLV lymphoid-transformed cell line are compared in Fig. 2. It is clear that the p53 molecules in the different primary tumors sharing size and antigenic determinants also have the same peptide map under these conditions. The p53 products of the primary tumors are indistinguishable from those in the established lines.

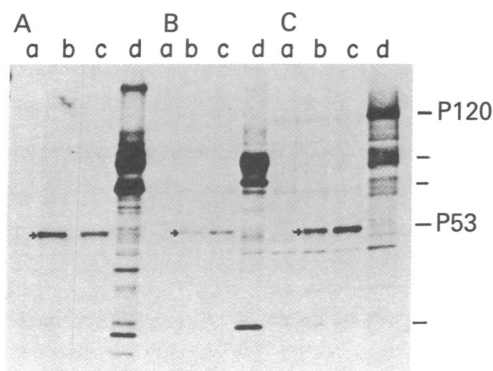Previous studies have shown that p53 can be detected in nor-

FIG. 1. Detection of the p53 molecule in induced and spontaneous primary mouse tumors. (*A*) The 147-800 spontaneous mammary adenocarcinoma of BALB/c origin. (*B*) The MT-6 spontaneous mammary adenocarcinoma of AKR origin. (*C*) The A-MuLV-transformed established cell line 2M3. Individual cell lysates were immunoprecipitated with normal serum (lanes a), RA3-2C2 (lanes b), PAb122 (lanes c), and goat anti-M-MuLV serum (lanes d).

mal thymocytes, although at very low concentrations (1, 2). Some of the primary tumors tested here consisted of tumor cell populations contaminated with a low percentage of normal thymocytes. It is therefore important to find a means to distinguish between the two by a parameter other than quantity.

It has been shown in other systems that transformation-related products are mostly found as phosphoproteins. For example, the 34- to 36-kilodalton protein described as a substrate for the *src* gene in avian sarcoma-transformed cells is phosphorylated in transformed cells but not in cells infected with a temperature-sensitive viral mutant. In the latter case, this protein can be metabolically labeled with [^{35}S]methionine (21, 22).

The p53 of established cell lines is a phosphoprotein and, under *in vivo* conditions, is phosphorylated on a serine residue (8, 14). I therefore tested whether the p53 of primary tumors and that of normal thymocytes are also phosphoproteins.

For that purpose, identical amounts of various cells [(*i*) 2M3/M; (*ii*) 2M3 A-MuLV transformed cell lines; (*iii*) 148-172 T-cell lymphoma; (*iv*) MT-6 mammary adenocarcinoma; (*v*) normal C57BL/6 thymocytes; (*vi*) normal BALB/c thymocytes; (*vii*) normal C57BL/6 spleen cells; and (*viii*) normal BALB/c spleen cells] were treated with ^{32}P$_i$ for 6 hr. Then, the cell lysates were immunoprecipitated with RA3-2C2 and PAb122 anti-p53 antibodies and electrophoresed on NaDodSO$_4$/polyacrylamide gels. In parallel, I labeled other aliquots of the same cells with [^{35}S]methionine and treated them as described above.

The results obtained are summarized in Table 2. Both the 2M3/M and the 2M3 cell lines, as well as the primary tumors tested (*iii* and *iv*, above), contained a p53 molecule that was phosphorylated under the present conditions. The p53 molecule of normal thymocytes, which is detected as a [^{35}S]methionine-labeled product, is not phosphorylated under the same conditions. Also, normal spleen cells with very low levels of [^{35}S]methionine-labeled p53 were not phosphorylated under the present conditions. In this case, the amount of [^{35}S]methionine-
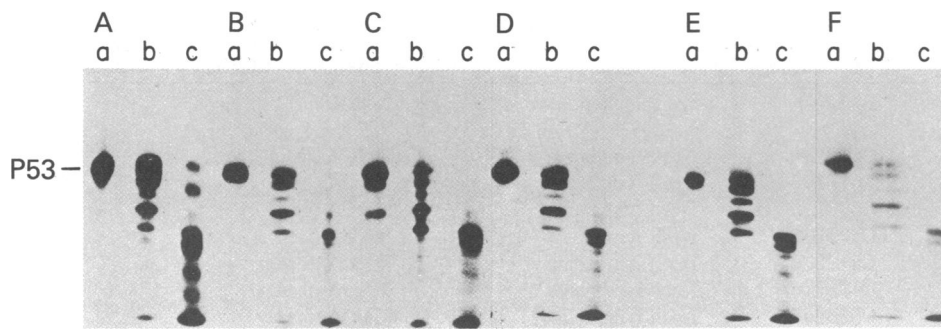
FIG. 2. Comparison of partial *S. aureus* V8 proteolytic digestion products of the p53 products from spontaneous and induced primary tumors with those from the A-MuLV-transformed cell line 2M3. (*A*) 148-181. (*B*) 148-193. (*C*) 148-50. (*D*) MT-6. (*E*) MT-147-800. (*F*) 2M3. Lanes: a, no enzyme; b, 10 ng of enzyme; c, 20 ng of enzyme.

labeled p53 from each tumor was compared with that in the normal thymus population. A comparison between the 148-172 chemically induced thymoma and normal thymocytes is shown in Fig. 3. In agreement with previous observations, normal thymocytes contain low amounts of immunoprecipitable [^{35}S]methionine-labeled p53. These cells did not exhibit any phosphorylated p53 immunoprecipitation with anti-p53 monoclonal antibodies. Phosphorylated p53 was detected in the 148-172 thymoma cell lysates under the same experimental conditions. These results suggest that the phosphorylated p53 molecule is found only in transformed cell lines and primary tumors.

DISCUSSION

The results presented here show that the p53 found in abundance in spontaneous and induced primary tumors is identical to that previously detected in various established cell lines (1–3). This p53 product is detectable in a wide range of primary tumors that includes T and B lymphomas and different sarcomas. The wide distribution of this protein in primary tumors suggests that it is a common transformation-related product and thus can serve as a tumor cell marker in mice. The fact that human cell lines contain the same molecule as that observed in mice (6, 7) suggests that the same antibodies could be used for the detection of the p53 marker in primary human tumors. The p53 synthesized in primary tumors was found in amounts comparable with those in established lines; the values were much higher than in normal mouse thymocytes. As in the established cell lines, the p53 of primary tumors was in the phosphorylated form. The p53 equivalent phosphorylated form was not de-

tected in the normal thymocyte population, suggesting that, at least in this respect, the p53 of transformed cells is distinguishable from that in normal cells. The fact that p53 accumulates in transformed cells regardless of the primary cause of transformation suggests that this is a common secondary event in the multistage process leading to the establishment of the malignant transformed cell. It should be mentioned, however, that p53 was not detected in spontaneous tumors of AKR mice. It is possible that this group of neoplastic cells lacks this protein or contains an equivalent molecule that is not recognized by the antibodies used in the present experiments. In this study, p53 was evaluated by measuring the amount of [^{35}S]methionine-labeled protein immunoprecipitating with specific monoclonal antibodies. Recently, however, a radioimmunoassay measuring total amounts of p53 has been developed (23). This approach will be useful for the analysis of total p53 amounts in mouse and human tumors.

The search for tumor-specific antigens in the last few years has mainly involved attempts to find such markers on the cell surface. Several such proteins have been described and they were mostly found to be specific for certain tumor types. The host-encoded protein p53 studied here is a cellular protein, not expressed on the cell surface. In A-MuLV-transformed lymphoid cells, p53 was detected mainly in the cytosol, not on the cell surface (8). In simian virus-transformed fibroblasts, the nonviral p53 was found mainly in the nucleus, where it was complexed with the large tumor antigen (11). Recently, it has been observed that, in chemically transformed Meth A fibroblasts, p53 is also concentrated in the nucleus and detected there as an unbound product (unpublished data). Although the p53

Table 2. Comparison of p53 molecules labeled with [^{35}S]-methionine and ^{32}P$_i$ from different cells

		p53 labeled	
	Mouse strain	Methionine, %	P$_i$, %
2M3/M A-MuLV cell line	BALB/c	398	154
2M3 A-MuLV cell line	BALB/c	402	183
147-172 T lymphoma	C57BL/6	321	100
MT-6 mammary adeno-carcinoma	BALB/c	274	236
Normal thymocytes	BALB/c	100	0
Normal spleen cells	BALB/c	12	0
Normal thymocytes	C57BL/6	112	0
Normal spleen cells	C57BL/6	14	0

The amount of [^{35}S]methionine-labeled p53 was compared with that found in normal thymocytes under the same experimental conditions. Evaluation of p53 was done as described in Table 1; results represent percentage of that observed in normal thymocytes. The amount of ^{32}PO$_4$-labeled p53 was compared with that found in tumor 147-172, which was designated 100%.
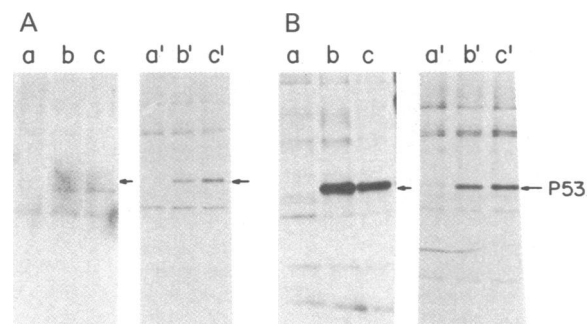

FIG. 3. Comparison of the chemically induced thymoma 148-172 and normal thymocytes. Aliquots of thymoma cells (*B*) and normal thymocytes (*A*) were labeled for 6 hr with ^{32}P$_i$ or [^{35}S]methionine. Cell lysates were prepared and equal amounts of phosphorylated (lanes a, b, c) or [^{35}S]methionine-labeled (lanes a', b', and c') proteins were immunoprecipitated with normal serum (lanes a and a') RA3-2C2 (lanes b and b'), or PAb122 (lanes c and c').

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molecule is not exposed on the cell surface, tumor-bearing mice develop anti-p53 antibodies, mainly at very late stages of tumor development. It is probable that, at this point, some of the tumor cells are lysed as a result of necrosis and the p53 is thus exposed to the immune lymphocytes, triggering antibody production (1).

The fact that the gene encoding p53 is well-conserved implies that the p53 protein fulfills a function in normal cells. Milner and Milner (24) have shown that p53 is induced in cells treated with mitogens and suggested that it starts functioning early during the transition from G_0 to G_m. Others have suggested that p53 is the R protein that has been shown to play a role in initiating events leading to DNA synthesis (25).

In addition to its presence in transformed cells, the p53 molecule was also found in embryonic cells (26, 27). It was detected in primary cultures of 12- to 14-day embryos, although in low amounts; cells of 16-day embryos seemed to have no p53 at all (26). A reduction in p53 level was also observed in F-9 embryonal carcinoma cells undergoing retinoic acid differentiation (28), suggesting that normal cell differentiation is accompanied by a reduction in the level of this protein. On the other hand, the level of p53 seems to increase in cells undergoing malignant transformation. It was observed that the tumor cell promoter phorbol 12-myristate 13-acetate induces expression of the p53 molecule in cells lacking this product, this being mediated through the acquisition of a fully transformed phenotype (29).

The fact that p53 is detected in both early undifferentiated cells and primary tumor cells supports the hypothesis that malignant transformation involves de-differentiation events.

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