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Coordinate expression of the endogenous p53 gene in β cells of transgenic mice expressing hybrid insulin–SV40 T antigen genes

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The expression of p53 has been evaluated during oncogenesis of the pancreatic β cells in transgenic mice harboring hybrid insulin–SV40 T antigen genes. Significant levels of p53 are detected in all cells expressing large T antigen. In contrast, the protein is undetectable in normal β cells. There is a complete correspondence between the onset of expression of T antigen and the appearance of the endogenous p53 protein. In tumors, the two proteins are found in a complex. In addition, free uncomplexed T antigen is detected in every cell which expresses the transgene. These results are consistent with the participation of p53 in T antigen-induced tumorigenesis *in vivo*. The early appearance of p53 in all β cells expressing large T cannot readily explain the progression of a small fraction of these cells into solid tumors.

Key words: insulinomas/oncogenesis/p53/SV40 T antigen/transgenic mice

Introduction

p53 is a cellular phosphoprotein (Crawford *et al.*, 1980; Jay *et al.*, 1980) present at high levels in a variety of cells transformed by viruses, chemicals and X-rays (for reviews see Crawford, 1983; Oren, 1985; Rotter and Wolf, 1985). In normal fibroblasts p53 has a short half-life of ~20 min, and is present at very low levels. In contrast, in cells transformed by SV40 or adenovirus its half-life is dramatically increased, to about 24 h (Oren *et al.*, 1981). This is achieved by formation of a stable non-covalent complex between p53 and the oncogene product, although there is evidence that free p53 is stablilized as well (Deppert and Haug, 1986). It has been suggested that p53 is synthesized at about the same rate in normal and transformed cells and that its expression is regulated mainly at the post-translational level (Rotter and Wolf, 1985). An increase in steady-state levels of p53 has been observed prior to DNA synthesis in cells stimulated by mitogens (Milner and Milner, 1981; Reich and Levine, 1984) and in quiescent cells after serum stimulation (Mercer *et al.*, 1984). DNA synthesis in these cells is inhibited by microinjection of a monoclonal antibody to p53 (Mercer *et al.*, 1982). The murine p53 can immortalize primary rodent cells when expressed at high levels (Jenkins *et al.*, 1984) and can cooperate with *ras* in transformation of these cells in a similar manner to *myc* or E1A (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). These results suggest an important role for p53 in the regulation of normal cell growth.

SV40 T antigen is capable of both adapting primary cells to continuous growth in culture and transforming cultured cells into a tumorigenic condition. Most known oncoproteins are proficient in one of these activities but not both (Bishop, 1985; Weinberg, 1985). It is possible that part of the activities of large T are mediated through its association with p53. The ability to target expression of T antigen to specific cell types in transgenic mice using cell-specific regulatory regions provides a system in which the expression of p53 can be examined at various stages during oncogenesis (for reviews see Hanahan, 1986; Palmiter and Brinster, 1986). We have previously reported (Hanahan, 1985; Efrat and Hanahan, 1987; Adams *et al.*, 1987) that transgenic mice harboring hybrid insulin–T antigen genes manifest heritable patterns of tumor formation. In all mice inheriting the hybrid oncogene, denoted RIP1–Tag, its cell-specific expression in β cells in the pancreatic islets of Langerhans results in hyperplasia of these cells, followed by development of a few islets into solid, vascularized tumors and consequent premature death. All the islets contain β cells expressing large T but only a few of the several hundred islets develop into tumors. Thus it appears that T antigen is necessary but not sufficient for tumor formation and that additional events may be required. The possibility that expression of the endogenous p53 gene may have a role in the progression from islets to tumors has been investigated. Here we report that high levels of p53 are detected in all β cells expressing T antigen, which argues against a role for p53 as a tumor progression factor in this system, but leaves open its possible participation in the actions of large T in β cell proliferation and transformation.

Results

Expression of p53 in β-cell tumors

The expression of p53 in β cells of RIP1–Tag transgenic mice was first characterized in the β-cell tumors, which represent a highly enriched population of cells expressing the transgene. These cells produce high levels of T antigen mRNAs and protein (Hanahan, 1985; Efrat and Hanahan, 1987). Northern blot analysis of tumor poly(A)$^+$ RNA reveals appreciable amounts of p53 mRNA in these cells (Figure 1). The relative abundance of p53 mRNA is at least one order of magnitude lower than T antigen mRNAs (Efrat and Hanahan, 1987), which are easily detectable in Northern analysis of total tumor RNA. The levels of p53 mRNA in tumors are comparable to those observed in normal mouse tissues (Figure 1), in agreement with previous reports (Rogel *et al.*, 1985; E.Harlow, personal communication). We were unable to establish the levels of p53 mRNA in normal β cells, since they comprise <1% of the pancreas.

Southern blot analysis has shown that neither the T antigen transgene nor the endogenous p53 gene are amplified or rearranged in the tumor cells when compared with other tissues of the transgenic mice (not shown), indicating that the p53 transcripts observed in the tumors are generated by the normal mouse p53 gene (Bienz *et al.*, 1984).

Immunoprecipitation of lysates from tumor cells, labeled *in vitro* with [^{35}S]methionine, reveals the presence of p53 protein

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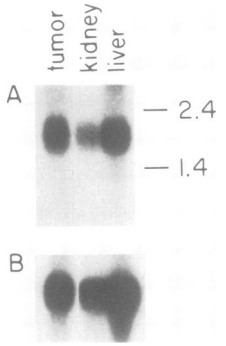

Fig. 1. Northern analysis of RIP1−Tag tumor mRNA. 5 μg of poly(A)$^+$ RNA were separated on a 1% formaldehyde−agarose gel, transferred to nitrocellulose and hybridized with a mouse p53 cDNA probe (**A**). The hybridized probe was then melted off and the blot rehybridized with a chicken β-actin probe, to control for relative mRNA levels and quality (**B**). The tumor is from an 8-month-old RIP1−Tag4 mouse. The kidney and liver mRNAs were prepared from a normal adult mouse. The sizes of the RNA markers are indicated in kb.

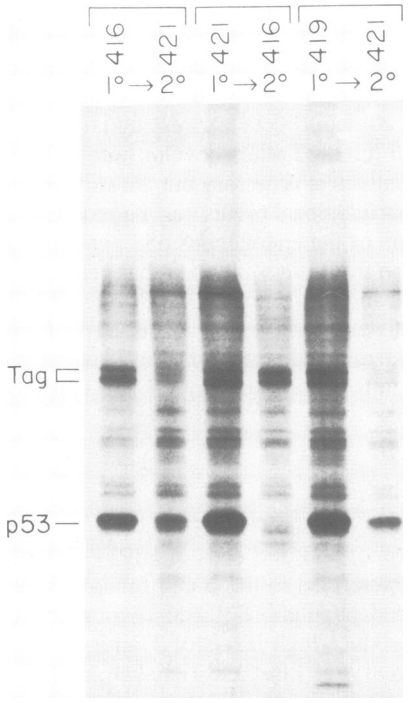

Fig. 2. Immunoprecipitation analysis of p53 and T antigen in RIP1−Tag2 tumor cells. Aliquots of lysate from cells labeled with [^{35}S]methionine, corresponding to 6 × 10^6 cells and containing 8 × 10^7 TCA-preciptable c.p.m., were immunoprecipitated with a 1:2 dilution of the monoclonal antibody indicated in the first lane (marked 1°) in each pair of lanes. The supernatants (unreacted material) of the immunoprecipitates were used for a second immunoprecipitation, with the monoclonal antibody indicated in the second lane (marked 2°) in each pair of lanes. The immunoprecipitates were then analysed on a 7.5−15% SDS−polyacrylamide gel.

(Figure 2). The monoclonal antibody PAb 421, directed against p53, coprecipitates T antigen as well. Similarly, each of the two monoclonal antibodies directed against T antigen (PAb 416 and PAb 419) also coprecipitates p53. (PAb 419 also precipitates the small t protein, observed at the bottom of the lane). This demonstrates that most of the large T and p53 are found complexed to each other in the tumor cells. When the supernatant from each primary immunoprecipitation was used in a second immunoprecipitation, this time with an antibody against the other protein, additional amounts of free, uncomplexed proteins were detected. Thus, after extensive immunoprecipitation of complexed p53 with either PAb 416 or PAb 419, a second precipitation with PAb 421 still detects considerable amounts of uncomplexed p53. Similarly, following with PAb 416 after immunoprecipitation with PAb 421 reveals the presence of free T antigen. Although the T antigen-mouse p53 complex appears to be remarkably stable (McCormick and Harlow, 1980), some dissociation may take place during the immunoprecipitation procedure. Therefore the ratio between complexed and free proteins observed by immunoprecipitation from lysates may differ somewhat from the actual situation inside the cells.

While the steady-state levels of p53 mRNA in the tumor β cells are considerably lower than those of T antigen messages, the observed abundance of the two proteins is comparable. This could be due to differences in the rates of translation or protein turnover. (There are twice as many methionine residues in large T compared to the mouse p53 protein, therefore the actual molar ratio between p53 and T antigen is even higher than indicated in this analysis).

Evaluation of p53 protein in normal and pre-neoplastic β cells

The high level of p53 observed in the β tumor cells is in agreement with the well-established presence of p53 in cultured cells transformed by SV40. However, these previous studies have not allowed for the examination of p53 during a 'pre-neoplastic' condition of T antigen expression, prior to the appearance of the fully transformed state. Nor has the role of p53 in cell transformation by T antigen been established. The transgenic β cells undergo a progression from a 'normal condition' to one of general proliferation (hyperplasia), to a less frequent one of neoplasia. An immunohistochemical analysis was performed, in order to assess the expression of p53 in β cells of mice at various ages, and thus determine the relative onset of expression of p53 and large T proteins, and to investigate the possibility that p53 is involved in the progression of some islets to tumors. Mice from two RIP1−Tag lineages, descending from two independent integration events of the transgene, were employed in this study. In the RIP1−Tag2 lineage, T antigen is expressed early during development. The islets of neonatal mice are normal, but by 6 weeks of age most of the islets are hyperplastic (and the animals slightly hypoglycemic). By 12−14 weeks, a few of the ~500 islets have progressed into solid, highly vascularized tumors (Hanahan, 1985). In contrast, in the RIP1−Tag4 lineage T antigen is first detected at 10−12 weeks of age (Adams *et al.*, 1987). These mice develop tumors around 8−9 months of age. An immunohistochemical analysis of T antigen and p53 expression in β cells of mice from these two lineages is presented in Figure 3. Both proteins show characteristic nuclear localization, as seen by the immunostaining. In the RIP1−Tag2 lineage T antigen is detected in neonatal islets and continues to be expressed in most of the β cells in all islets from adult mice and in tumor cells. p53 appears to follow a similar pattern of expression. It is clearly detected in neonatal β cells, in all the adult islets and in the tumors. When non-transgenic mice (C57B1/6J × DBA/2J) were similarly examined, p53 was not detectable in islets from neonatal nor from adult mice. Similarly, no p53 is detected in neonatal RIP1−Tag4 islets, which also do not express T antigen. In the islets of 12-week-old RIP1−Tag4 mice, p53 is clearly visible in a fraction of the cells, in concordance with the onset of T antigen expression. In this lineage the expression of T antigen

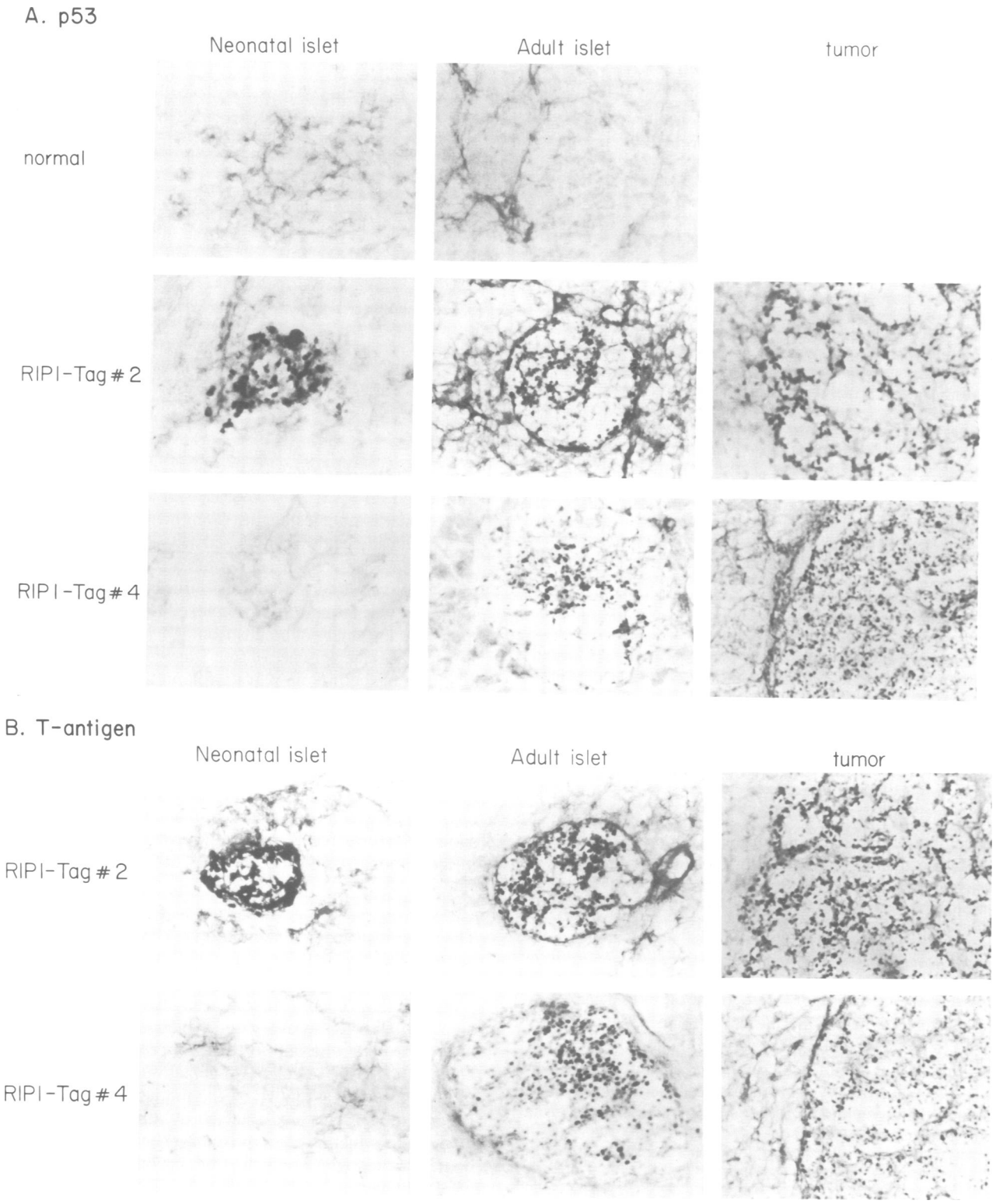

Fig. 3. Immunohistochemical analysis of p53 and large T antigen in the pancreas of RIP1−Tag mice. (**A**) Thin sections of pancreas were incubated with rabbit anti-p53 serum (for neonatal mice) or with a 1:1 mix of PAb 242 and PAb 246 (for adult mice), followed by visualization with peroxidase-conjugated second antibody. Neonatal islets are from day 1 newborns. Adult islets are from 4-week-old (RIP1−Tag2) and 12-week-old (RIP1−Tag4) mice. Tumors are from 12-week-old (RIP1−Tag2) and 9-month-old (RIP1−Tag4) mice. Magnification is × 162. (**B**) Adjacent sections of the tissues in **A**, stained with an anti-T antigen monoclonal antibody (PAb 416).

at 12 weeks occurs in a fraction of β cells, most of which are often clustered at one pole of the islet. The expression of p53 follows the same pattern. Both proteins are clearly expressed in RIP1−Tag4 tumors. These results demonstrate an identical pattern of expression of the two proteins in β cells from the two lineages. Every islet which expresses T antigen also contains p53. No islets are found which have β cells expressing only one of the two proteins.

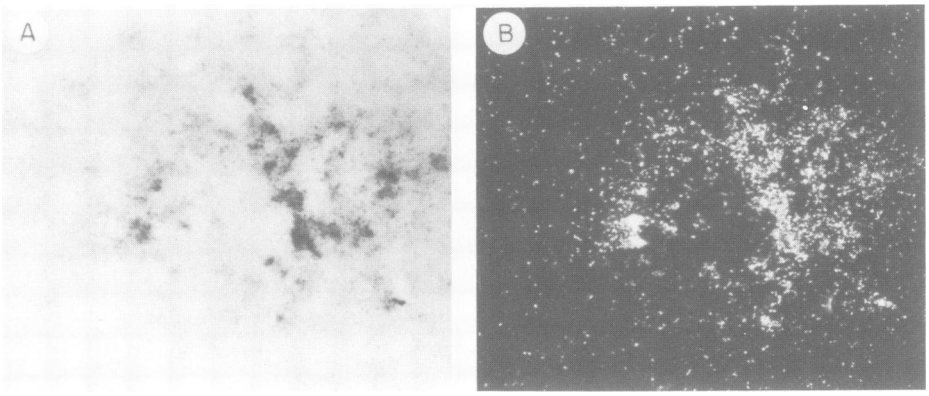

Fig. 4. Double-labeling of T antigen and p53 in 12-week-old RIP1−Tag4 pancreas. Sections were incubated with rabbit anti-T antigen serum, followed by anti-rabbit IgG labeled with ^{125}I. They were then incubated with rabbit anti-p53 serum, which was visualized by peroxidase-conjugated second antibody (seen as darkly stained areas). (No staining was obtained in control sections, in which the anti-p53 antibody was omitted). The sections were then coated with photographic emulsion, exposed for 3 weeks and developed to visualize the anti-T antigen antibody (seen as fine silver grains). Magnification is ×250. (**A**) bright field photograph; (**B**) dark field photograph.

Table I. Presence of uncomplexed large T protein in β cells

Age (months)	Normal (control)	RIP1−Tag2 Islets	RIP1−Tag2 Tumors	RIP1−Tag4 Islets	RIP1−Tag4 Tumors
1	−	+		−	
3	−	+	+	+	
9				+	+

Adjacent sections of pancreas were incubated with either PAb 413 or PAb 414 followed by a peroxidase-conjugated second antibody. Similar results were obtained with either antibody in each case. The blank points are not applicable.

Pancreatic islets isolated from both neonatal and adult normal mice (non-transgenic C57B1/6J × DBA/2J) were labeled *in vitro* and subjected to immunoprecipitation with anti-p53 antibodies. Using up to 500 islets (~2.5×10^5 cells) for each immunoprecipitation, with an amount of incorporated radioactivity comparable to that obtained with the tumor cells, we were unable to detect p53 in normal, untransformed β cells. In contrast, when islets were isolated from RIP1−Tag2 and RIP1−Tag4 mice, both p53 and T antigen were detected (data not shown). These results confirm those obtained by immunostaining, and indicate that the levels of p53 protein in normal β cells are either exceedingly low, or non-existent.

To show unequivocally that T antigen and p53 proteins are expressed in the same cells within a given islet, a double labeling experiment was performed (Figure 4). Sections of pancreas from 12-week-old RIP1−Tag4 mice were incubated sequentially with anti-T antigen and anti-p53 antisera, which were visualized using two different second antibodies. The results demonstrate that p53 (visualized by the peroxidase reaction) is expressed only in the cells staining for T antigen (marked by the fine grains). The double-labeled cells are clustered in one side of the islet, a pattern similar to that observed in the RIP1−Tag4 islets in Figure 3, which were stained separately with each antibody. We conclude that both proteins are expressed in the same cells in these islets.

Immunohistochemical analysis of uncomplexed T antigen

In transfection experiments T antigen can immortalize and transform primary cells without exogenous cooperating oncogenes such as *myc* or *ras*. One possible explanation for that is that several functions, normally controlled by separate oncogene products, are performed by different forms of the large T protein. Lane and Gannon (1986) have suggested that free T antigen, uncomplexed to p53, is necessary for transformation. Using monoclonal antibodies against T antigen that do not react with the protein molecules complexed with p53, they were able to detect free T antigen in cells transformed by SV40 but not in cells abortively infected with the virus. In the latter case all the T antigen was found to be complexed to p53. We have used *in situ* immunostaining of β cells from RIP1−Tag mice at different ages to assess the presence of free T antigen, to determine whether this form of the protein plays a role in the progression of islets to tumors. As summarized in Table I, free T antigen could be detected in all the islets expressing T antigen as well as in the tumors of mice from both the fast and slow lineage. These results indicate that in all β cells expressing T antigen the protein is found both in complex with p53 and in the uncomplexed form. This confirms the results obtained by immunoprecipitation, which has shown that both free T antigen and free p53 proteins are present in the tumor β cells (Figure 2).

Discussion

The data presented here demonstrate a complete correspondence between the targeted expression of T antigen to β cells of transgenic mice and the appearance of the endogenous murine p53 protein in these cells. A pronounced increase in endogenous p53 levels is observed in every cell which expresses T antigen. In the tumor cells, the bulk of the two proteins is found in complex, although these cells also contain uncomplexed molecules of both proteins, as shown by the immunoprecipitation studies. Free T antigen was also detected by immunohistochemistry in all the cells which express the transgene, using monoclonal antibodies that recognize only the free, uncomplexed protein. (Since we do not have an antibody that reacts specifically with free p53, it was not possible to confirm its presence in islet β cells by immunohistochemical analysis).

These results suggest that differences in free T and in p53 cannot simply account for the observed progression of normal islets to hyperplastic islets, to infrequent solid tumors. It seems likely that other secondary events are required to complement the activities of T antigen (either free or in association with p53) in inducing tumorigenesis. Nevertheless, p53 may have an important role in β cell proliferation and transformation. To investigate this possibility further, we have generated transgenic mice harboring insulin-promoted p53 genes.

The pronounced increase in p53 levels most probably results from post-translational stabilization of the protein through its association with T antigen, as is observed in cultured cells transformed by SV40 (Oren *et al.*, 1981). However, an effect of T antigen on transcription of the p53 gene or stability of the mRNA cannot be excluded, given the inaccessibility of sufficient numbers of normal β cells to permit biochemical analyses.

The observation that p53 protein is not detectable in normal, untransformed β cells from both neonatal and adult mice is perhaps not surprising, given its implied role in proliferation. Only a few percent of β cells are observed to be dividing in islets after birth as revealed by thymidine labelling (see Hellerstrom, 1984 for review; also Teitelman, Alpert and Hanahan, in preparation). The ability to increase the pool of proliferating β cells appears to be important in cases of increased insulin demand, such as in pregnancy and diabetes (Coleman, 1978). The appearance of p53 in proliferating β cells in the RIP1−Tag mice suggests that expression of p53 may be involved in recruiting β cells from a quiescent state into cell cycle in such cases.

Recently we have generated transgenic mice in which expression of T antigen is targeted to two of the other cell types in the islets of Langerhans, the α and δ cells, using the glucagon and somatostatin promoters, respectively (unpublished results). In these mice too, p53 is detected in the specific cell type as soon as expression of T antigen is observed. These findings confirm the results detailed here, and support the possibility that p53 is directly involved in cell transformation mediated by T antigen *in vivo*.

Materials and methods

Transgenic mice

Construction of the hybrid insulin-T antigen genes and generation of transgenic mice have been described previously (Hanahan, 1985).

Antibodies

The monoclonal antibodies PAb 413, 414, 416, 419 and 421 (culture medium) are from E.Harlow (Harlow *et al.*, 1981). PAb 413 and 414 recognize only free T antigen molecules, uncomplexed to p53 (Lane and Gannon, 1986). PAb 416 and 419 react with both complexed and free T antigen (PAb 419 also recognizes the small t protein). PAb 421 is specific for p53, as are the two monoclonal antibodies PAb 242 and PAb 246 (ascites fluid). PAb 246 binds preferentially to p53 in complex with T antigen, while PAb 242 recognizes equally well both complexed and free p53 (Yewdell *et al.*, 1986). The antiserum against T antigen was generated by immunizing rabbits with SV40 T antigen purified from COS cells. Anti-mouse p53 serum was raised by immunizing rabbits with a p53-β-galactosidase fusion protein isolated from *E. coli* using preparative SDS gel electrophoresis (Lane *et al.*, 1985).

Isolation of islets and tumor cells

Pancreatic islets were isolated by sequential collagenase incubations followed by centrifugation on a discontinuous Percoll (Pharmacia) gradient and selection under a stereo-microscope (Steffes *et al.*, 1981). Islets were cultured before labeling overnight in RPMI 1640 (GIBCO), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary cultures were prepared from freshly excised tumors by gentle mincing and filtration through a nylon mesh (600 μm) in the same medium. After sedimentation for 1 min to remove large fibrous clumps, the supernatant was transferred to a new tube and cells were sedimented for 10 min in 10 ml of the culture medium. The supernatant was collected in four fractions and the cells in those fractions, as well as those in the sediment, were washed by centrifugation for 5 min at 50 *g*. Typically, the sediment and the bottom 1−2 fractions were collected, whereas the top fractions, containing mostly dead tumor cells and erythrocytes, were discarded. Cells were incubated 1−2 h in culture medium at 5×10^5 cells per ml and then washed in methionine-free medium prior to labeling.

In vitro labeling of cells and immunoprecipitation

Islets (500/ml) were labeled with [^{35}S]methionine for 4 h in methionine-free RPMI 1640 medium, supplemented with 2% Nu-serum (Collaborative Research) and 0.5% heat-inactivated human serum. Tumor cells were labeled in the same medium at $1-5 \times 10^6$ cells per ml in spinner culture flasks for 4 h. Islets or tumor cells were lysed in a buffer containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM phenylmethylsulfonylfluoride (Boeringer-Mannheim), 0.5% Trasylol (NOVO Industries, Bagsvaerd), 5 mM EDTA, 0.1 mM *p*-chloromercuribenzenesulfonic acid and 1% NP-40 for 1 h (for tumor cells) or 2 h (for islets). After centrifugation at 100 000 *g* for 30 min, aliquots of the lysate were pre-cleared with non-immune serum and immunoprecipitated as described (Baekkeskov, 1984). Immunoprecipitates were processed and analysed by SDS−PAGE and fluorography as detailed (Baekkeskov *et al.*, 1987).

Immunohistochemistry

The sectioning of fresh frozen tissue and immunohistochemical visualization of antigens with horseradish peroxidase-conjugated antibodies were detailed elsewhere (Hanahan, 1985; Efrat and Hanahan, 1987; Adams *et al.*, 1987). The PAb 413−421 monoclonals were used undiluted (as tissue culture medium). PAb 242 and PAb 246 were used at a 1:50 dilution. Anti-T antigen and anti-p53 serums were diluted 1:5000 and 1:1000, respectively and visualized with a 1:200 dilution of peroxidase-conjugated goat anti-rabbit IgG (Accurate Chemical and Scientific Corp.). For double-labeling experiments, sections were incubated overnight with anti-T antigen serum diluted 1:500 000 (a dilution which does not allow detection with the peroxidase-conjugated second antibody), followed by washes in 1% goat serum in PBS and 30 min incubation in a 1:50 dilution of donkey anti-rabbit IgG labeled with ^{125}I (spec. act. 5−20 μCi/μg, Amersham). The sections were washed and incubated with anti-p53 serum, followed by washes and second antibody incubation, as in single-labeling experiments. The sections were washed in 0.1 M Tris, pH 7.5 and 0.9% sodium chloride and the bound peroxidase was visualized by a 6 min reaction with 0.5 mg/ml diaminobenzidine and 0.003% H_2O_2 in 10 mM Tris, pH 7.2. The sections were then washed, fixed for 10 min with 4% paraformaldehyde in PBS, washed in water, dehydrated through graded alcohols and air-dried. The sections were then dipped in Ilford L-4 photographic emulsion diluted 1:1 in water, air-dried and exposed at 4°C. After 3 weeks the sections were developed for 3 min at 15°C in Kodak D-19 developer, rinsed in water, fixed for 10 min at 15°C in Kodak Ektaflo fixer, dehydrated and mounted with Permount.

RNA analysis

Isolation of RNA and Northern blotting analysis have been dscribed (Efrat and Hanahan, 1987). Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT) columns (Aviv and Leder, 1972). RNA size markers (Bethesda Research Laboratories) were visualized by u.v. transillumination following staining with ethidium bromide.

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