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Loss of p53 function through PAX-mediated transcriptional repression

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Direct interactions between the genes that regulate development and those which regulate the cell cycle would provide a mechanism by which numerous biological events could be better understood. We have identified a direct role for PAX5 in the control of p53 transcription. In primary human diffuse astrocytomas, PAX5 expression inversely correlated with p53 expression. The human p53 gene harbours a PAX binding site within its untranslated first exon that is conserved throughout evolution. PAX5 and its paralogues PAX2 and PAX8 are capable of inhibiting both the p53 promoter and transactivation of a p53-responsive reporter in cell culture. Mutation of the identified binding site eliminates PAX protein binding *in vitro* and renders the promoter inactive in cells. These data suggest that PAX proteins might regulate p53 expression during development and propose a novel alternative mechanism for tumour initiation or progression, by which loss of p53 function occurs at the transcriptional level.

Keywords: cancer/development/PAX/p53 transcription

Introduction

One aim of genes that are involved in controlling developmental processes is to influence the cell cycle in time and space. By manipulating the cell cycle, developmental regulatory genes direct cellular growth and differentiation, and thus permit embryogenesis to proceed in an exquisitely controlled manner. Such genes are also likely candidates for involvement in oncogenesis, owing to their effects on cell growth.

Pax genes encode nuclear transcription factors which have been implicated in the control of mammalian development (Gruss and Walther, 1992; Chalepakis *et al.*, 1993; Stuart *et al.*, 1994). Thus far, nine mammalian *Pax* genes have been identified (Stapelton *et al.*, 1993). All PAX proteins contain a conserved DNA binding motif termed the paired box. In addition, some contain a full homeodomain (PAX3, 7, 4 and 6) whereas others contain a partial homeodomain (PAX2, 5 and 8) and yet others none at all (PAX1 and 9). Mutations within *Pax* genes have been demonstrated to be involved in three mouse developmental mutants and two corresponding human syndromes (Chalepakis *et al.*, 1993; Stuart *et al.*, 1994). During development of the central nervous system, PAX2, 5 and 8 are most highly expressed in the ventricular zone. In addition, PAX5 is an important regulator of B cell development and its mutation arrests B cells at an early stage of development (Barberis *et al.*, 1990; Adams *et al.*, 1992; Urbanék *et al.*, 1994). Murine *Pax* genes have been shown to act as proto-oncogenes as they are able to transform cells in culture which subsequently form tumours in nude mice (Maulbecker and Gruss, 1993). Recently, a number of human *Pax* genes have been implicated in various types of cancer—*Pax5* in astrocytomas (Stuart *et al.*, 1995), *Pax2* and *8* in Wilms' tumour (Dressler and Douglass, 1992; Poleev *et al.*, 1992) and *Pax3* and *7* in rhabdomyosarcoma (Barr *et al.*, 1993; Galili *et al.*, 1993; Shapiro *et al.*, 1993; Davis *et al.*, 1994).

The p53 tumour suppressor gene encodes a nuclear transcription factor and is the most commonly mutated gene in human cancer thus far identified (Oren, 1992; Donehower and Bradley, 1993). These mutations commonly occur in its DNA binding domain, rendering it unable to transactivate its target genes (Prives, 1994). p53 appears to control the cell cycle by arresting cells in late G_1 phase by activating downstream target genes, notably *WAF1* (El-Deiry *et al.*, 1993). In addition to its role in the cell cycle, p53 has been demonstrated to mediate apoptosis in a variety of cell types (Oren, 1994). Studies involving the control of expression of p53 itself have so far provided only limited insight into the transcriptional regulation of the p53 gene (Zambetti *et al.*, 1992; Deffie *et al.*, 1993). In contrast, the p53 protein appears to be regulated exquisitely by a variety of mechanisms which affect its quantity as well as its function (Momand *et al.*, 1992; Oliner *et al.*, 1992; Zauberman *et al.*, 1993) and also by viral proteins (Zambetti *et al.*, 1992; Levine, 1993a,b).

The role of p53 in developmental processes has not been studied extensively, although it appears to undergo temporal and spatial changes in expression during prenatal development (Rogel *et al.*, 1985; Schmid *et al.*, 1991).

In this report, we demonstrate that one class of PAX proteins is capable of inhibiting human p53 expression by direct DNA binding of the PAX protein to a sequence in the 5′ region of the p53 gene. In addition, mutational analysis of the PAX binding site revealed that it is absolutely required for basal promoter activity, suggesting that PAX proteins may bind to this site and physically impede an as yet unidentified positive regulatory factor transactivating the promoter. We discuss potential implications of these results for oncogenesis and we present a model for PAX-mediated regulation of the cell cycle and of specific differentiation events during embryonic development.

Results

PAX5 expression in human astrocytomas

We have previously demonstrated a positive correlation between PAX5 expression and the level of malignancy in

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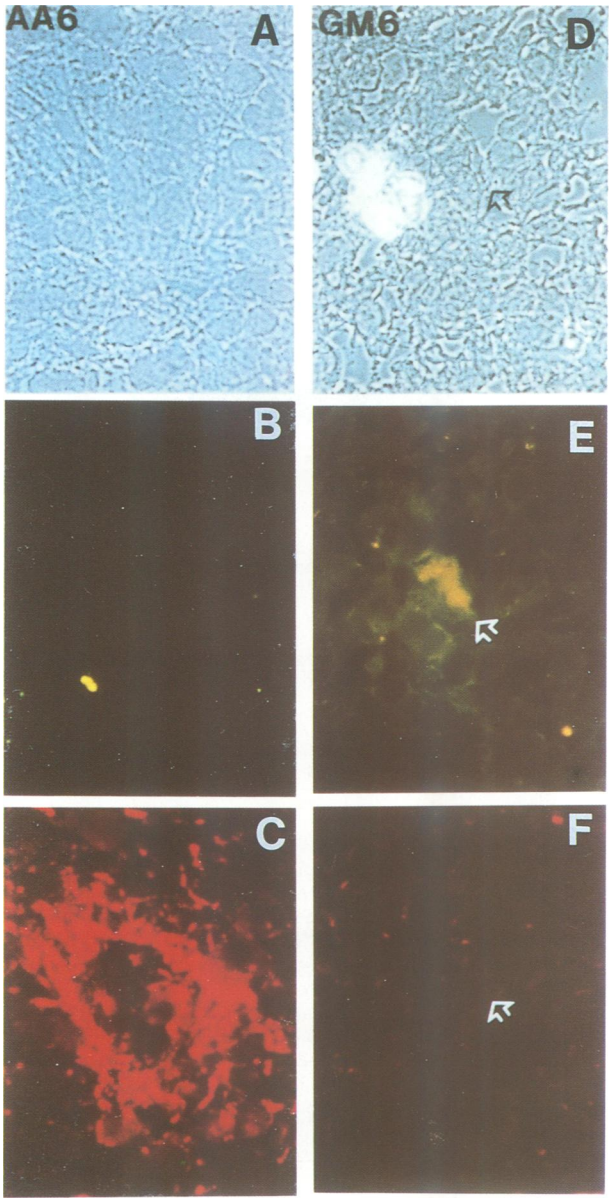

Fig. 1. PAX5 mRNA expression inversely correlates with mutant p53 protein in primary human astrocytomas. In the left panel, no PAX5 expression was detected in a medium grade (WHO grade III) anaplastic astrocytoma (**B**) and p53 was abundantly expressed (**C**). In the right panel, a representative glioblastoma multiforme (WHO grade IV), PAX5 was focally expressed (**E**) and p53 was not expressed (arrow) (**F**). Tumour identification numbers in the top left of each phase contrast panel correspond to those previously described (Stuart *et al.*, 1995).

human astrocytic tumours (Stuart *et al.*, 1995). In all cases, expression of PAX5 coincided with that of various oncogenes; however, when double labelling experiments were performed for PAX5 mRNA and p53 protein (PAb 240), it was evident that in low-grade astrocytomas where PAX5 was not expressed, p53 was highly expressed. While not formally proven, this overexpressed p53 is likely to be mutant. Alternatively, in highly malignant astrocytomas (glioblastoma multiforme), those areas which abundantly expressed PAX5 were consistently p53 negative (Figure 1 and data not shown). This observation appears to be specific for tumours which express PAX5, as a non-diffuse astrocytoma not expressing PAX5 but expressing PAX4

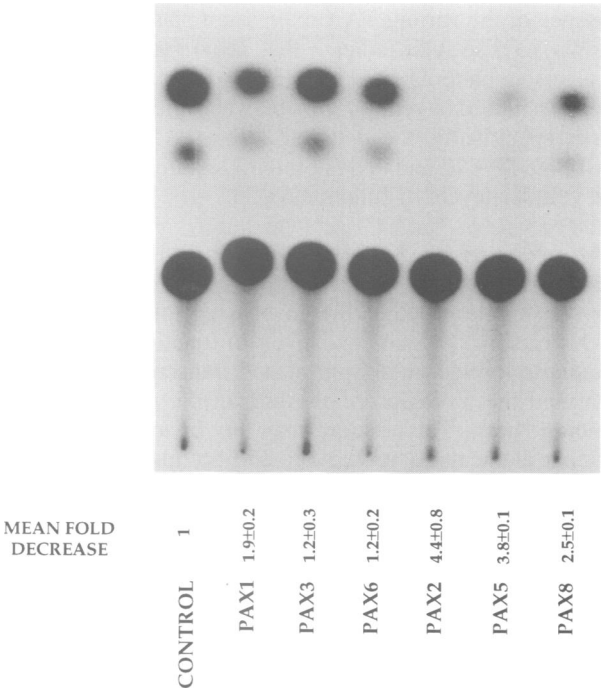

Fig. 2. PAX protein expression inhibits p53-mediated transactivation in transiently transfected NIH 3T3 cells. PAX 2, 5 and 8 decrease reporter activity, whereas other PAX proteins (which do or do not contain a homeodomain) are unable to do so.

did not show a negative correlation between PAX4 and p53 (data not shown). As the stability of mutant p53 protein is typically much greater than that of wild-type p53, we cannot rule out the possibility that wild-type p53 was present but not detectable in tumour cells over-expressing PAX5. However, our data raised the possibility that PAX5 may influence p53 expression or function, either directly or indirectly.

PAX2, 5 and 8 modulate p53-dependent reporter activity

In an attempt to investigate a possible relationship between PAX5 and p53, we studied the effect of PAX proteins on the activity of endogenous p53 in cell culture. To that end, NIH 3T3 mouse fibroblasts were co-transfected with the RGC–CAT reporter plasmid which contains multiple copies of a p53 binding sequence in front of the chloramphenicol acetyl transferase (CAT) gene together with various PAX expression plasmids. Figure 2 shows that PAX5 had a pronounced inhibitory effect on p53-dependent reporter activity. A comparable inhibitory effect was also exerted by PAX2 and PAX8, both of which share substantial sequence and structural similarity with PAX5 (Stuart *et al.*, 1994). PAX1 and PAX6 expression had a partial inhibitory effect, whereas PAX3 did not affect the transcriptional activity of p53 at all. Variations in the relative inhibitory effect of each PAX were observed, yet the overall pattern emerging from multiple transfections is consistent with the data presented in the figure shown. As a control, an unrelated promoter–reporter construct (TK–CAT), which contains the thymidine kinase promoter linked to CAT, was employed to test whether the reduction in reporter activity was due to PAX-induced transcriptional squelching. No difference was seen in the presence or

absence of the various PAX expression plasmids (data not shown). These data indicate that, as suggested from the expression patterns of PAX5 and p53 in tumour cells, there is indeed a relationship between the two proteins. More specifically, the data show that PAX5, as well as the related PAX2 and PAX8 proteins, can markedly reduce the cellular levels of functional p53.

PAX proteins bind to a sequence within exon 1 of p53

To investigate whether the inverse correlation between PAX protein expression and levels of functional p53 could be due to a direct inhibition of p53 transcription, we first analysed the 5′ region of the human p53 gene for PAX binding sites. This region may be divided into three distinct elements: a stretch of ~250 bp upstream of exon 1 contains promoter activity (Tuck and Crawford, 1989), exon 1 and a large (10 kb) intron which harbours a strong but unrelated promoter in its first 1.2 kb (Reisman *et al.*, 1988). The p53 promoter, exon 1 and 1.2 kb 3′ of intron 1 were analysed for PAX binding sites by electrophoretic mobility shift assays (EMSAs). A single PAX binding site was identified which resides at the 3′ end of exon 1 between nucleotides +166 and +216 [+1 refers to the transcriptional start site, as previously defined (Tuck and Crawford, 1989)]. This binding site (Figures 3B and 4A) contains the PAX core motif GTYMC as well as the CA dinucleotide previously identified in PAX5 binding sites (Czerny *et al.*, 1993). Figure 3A shows binding of various PAX proteins to this sequence. PAX2, 5 and 8 bound most efficiently to this site, PAX1 and 6 exhibited relatively weaker binding, whereas PAX3 did not bind at all. The presence of a partial homeodomain in PAX2, 5 and 8 may be important for efficient binding, as two deletion mutants, *PaxΔ3* (similar to the murine *splotch Pax3*) and *PaxΔ6* (similar to the *small eye Pax6*), which contain only the paired domain, display greater binding to this sequence than their wild-type counterparts which contain a full homeodomain.

The sequence of the PAX binding site identified in the EMSA is highly conserved between mouse, rat and man (Figure 3B), supporting the functional significance of this site.

To characterize the PAX binding site further, we subjected it to mutational analysis (Figure 4A). Point mutation of the C within the CA dinucleotide did not affect binding; nor did mutation 4 (data not shown). By contrast, mutations 2 and 3, introduced within the PAX core motif, significantly reduced binding of all PAX proteins (Figure 4B).

PAX2, 5 and 8 inhibit the activity of the isolated p53 promoter in cell culture

PAX proteins bind to a sequence within the first exon of the human p53 gene, and PAX2, 5 and 8 are capable of strongly decreasing p53 protein function in cell culture. These observations proposed a mechanism whereby PAX proteins may exert their inhibitory effect on p53 function by directly repressing the transcription of the p53 gene itself. We therefore constructed a reporter plasmid, p53LUC, which contains a 490 bp genomic fragment comprising the p53 promoter and exon 1, in front of the firefly luciferase gene (Figure 5A, upper panel). This construct was co-transfected with PAX2, 5 and 8 expression plasmids into primary rat embryo fibroblasts and reporter activity was assayed (Figure 5A, lower panel). It is evident that PAX2, 5 and 8 virtually abolish p53 promoter activity. The inhibition of this p53 promoter construct by overexpression of PAX proteins argues strongly in favour of the contention that PAX proteins can serve as negative regulators of p53 expression, presumably through sequence-specific DNA binding to a cognate regulatory element in the p53 gene.

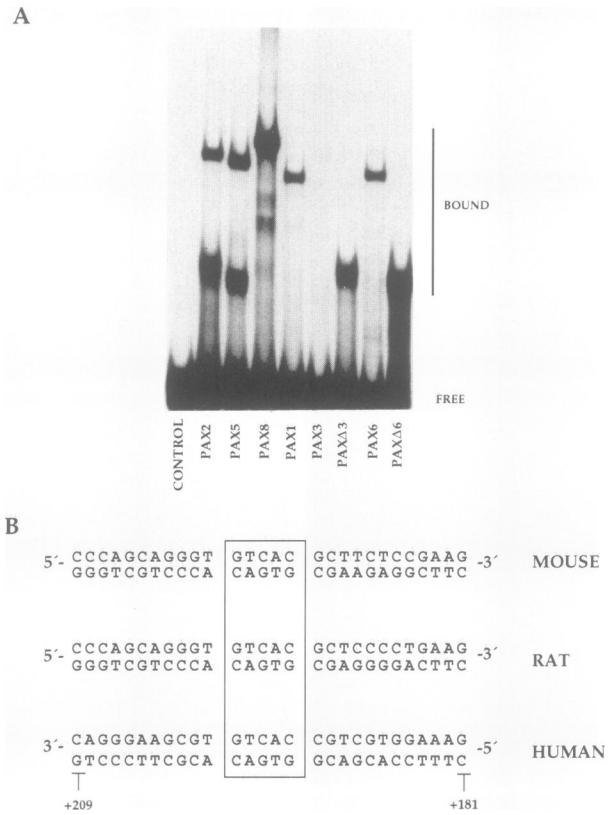

Fig. 3. PAX proteins bind to a sequence within the 3′ end of exon 1 of the human p53 gene. (**A**) EMSA was performed as described in Materials and methods. PAX2, 5 and 8 bind to a similar extent, PAX1 and 6 bind less efficiently and PAX3 not at all. PAXΔ3 and PAXΔ6 represent truncated proteins consisting of the paired domain only (see text). The PAX3 paired domain alone is able to bind weakly, whereas the PAX6 paired domain alone binds very strongly. (**B**) Evolutionary comparison of the sequence surrounding the PAX binding site within the first exon of the mouse, rat and human p53 genes. Sequences are from Bienz-Tadmor *et al.* (1985). In the mouse and rat, the GTCAC motif is located on the sense strand, whereas the human GTCAC motif is located on the antisense strand. Numbers refer to the nucleotide position according to Tuck and Crawford (1989).

Point mutation of the p53 promoter abolished transcriptional activity

To test whether the PAX binding site is an absolute requirement for transcriptional inhibition, we generated p53mutLUC (Figure 5B, upper panel). This reporter construct is identical to the wild-type p53 promoter–luciferase construct, with the exception of a single point mutation in the PAX binding site core motif previously found to virtually eliminate PAX binding (Figure 4A). Surprisingly, all promoter activity was totally ablated when this construct was transfected into a number of cell types, including NIH 3T3 and primary rat embryo fibroblasts (Figure 5B,

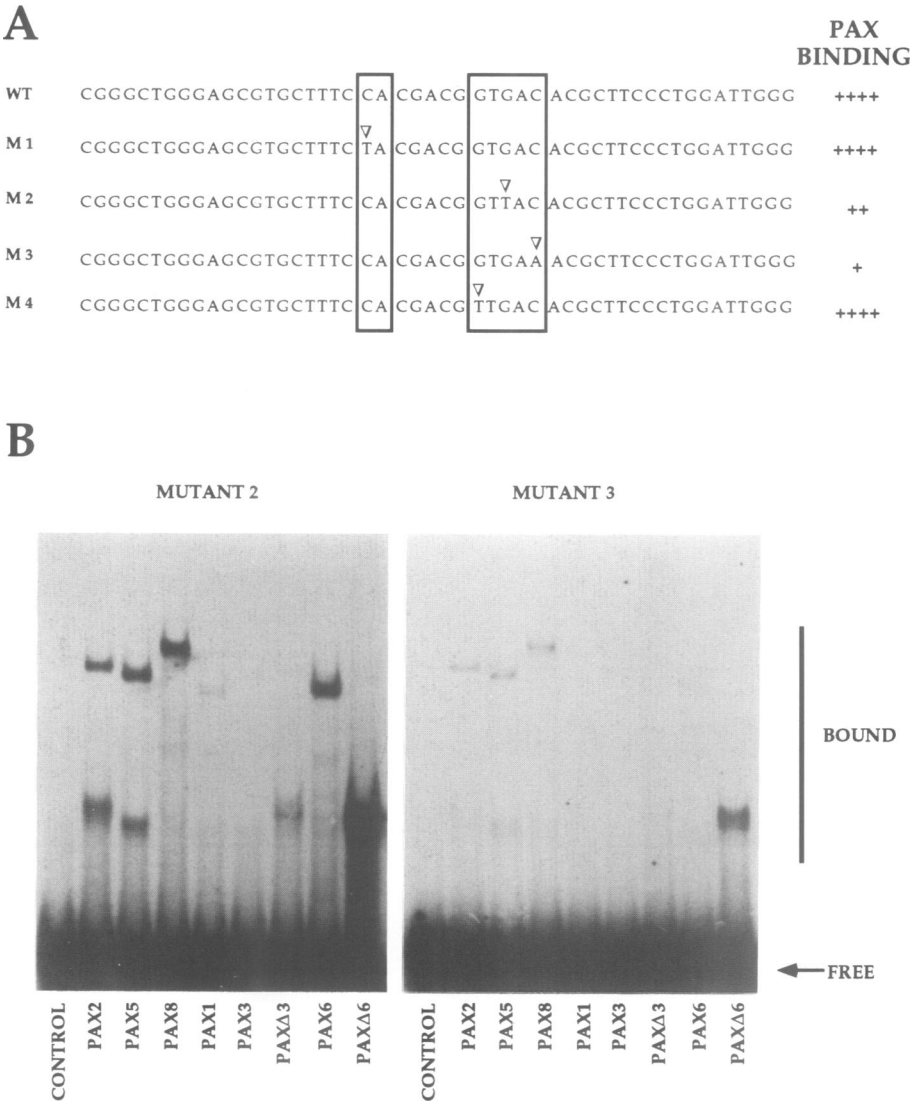

Fig. 4. (**A**) Point mutation of the pentanucleotide within the PAX binding site abrogates PAX protein binding *in vitro*. Oligonucleotides containing the point mutations shown (M1–M4) were synthesized and EMSA were performed as described in Materials and methods. The relative binding of PAX proteins was measured by phosphorimagery. (**B**) Point mutations within the pentanucleotide significantly reduce PAX protein binding. EMSA were performed and exposed to X-ray film for times similar to those shown in Figure 3.

lower panel). We conclude that the PAX binding site is also required for basal promoter activity. In support of this conclusion, deletion analysis of the p53 promoter and exon 1, from either the 5′ or 3′ end, demonstrated that only the most 3′ 85 bp of exon 1 were essential for promoter activity (Tuck and Crawford, 1989). Furthermore, the sequence directly 3′ to the PAX pentanucleotide core binding sequence is highly conserved between mouse, rat and human (Bienz-Tadmor *et al.*, 1985). This sequence, CACGCTTCCC, shows high homology to the consensus binding sequence for TFII-I, a regulator of basal transcription (Roy *et al.*, 1991). Our present data demonstrate that the cytosine at position +204 is a key determinant of this promoter activity. Presumably it constitutes a base contact for a factor governing p53 promoter activity. The importance of this cytosine to both PAX binding and promoter activity strongly suggests that PAX proteins target directly a DNA element which is essential for the activity of the p53 promoter. Such PAX–DNA interaction is likely to underlie the repression of p53 promoter activity by PAX5 and its relatives.

Discussion

We have demonstrated that PAX5 and its relatives are capable of inhibiting the function of p53. The mechanism by which this inhibition occurs is likely to involve PAX protein–DNA interactions at a site which is located close to one which is responsible for basal promoter activity.

Implications for developmental mechanisms

The identification of p53 as a downstream target gene for PAX proteins leads to the possibility of explaining some questions regarding the mechanism of action of PAX2, 5 and 8 during embryonic development. A theoretical model for PAX–p53 functional interactions is presented in Figure 6. In this theory, we propose the following mechanism of action. Early in embryonic development, a cell population

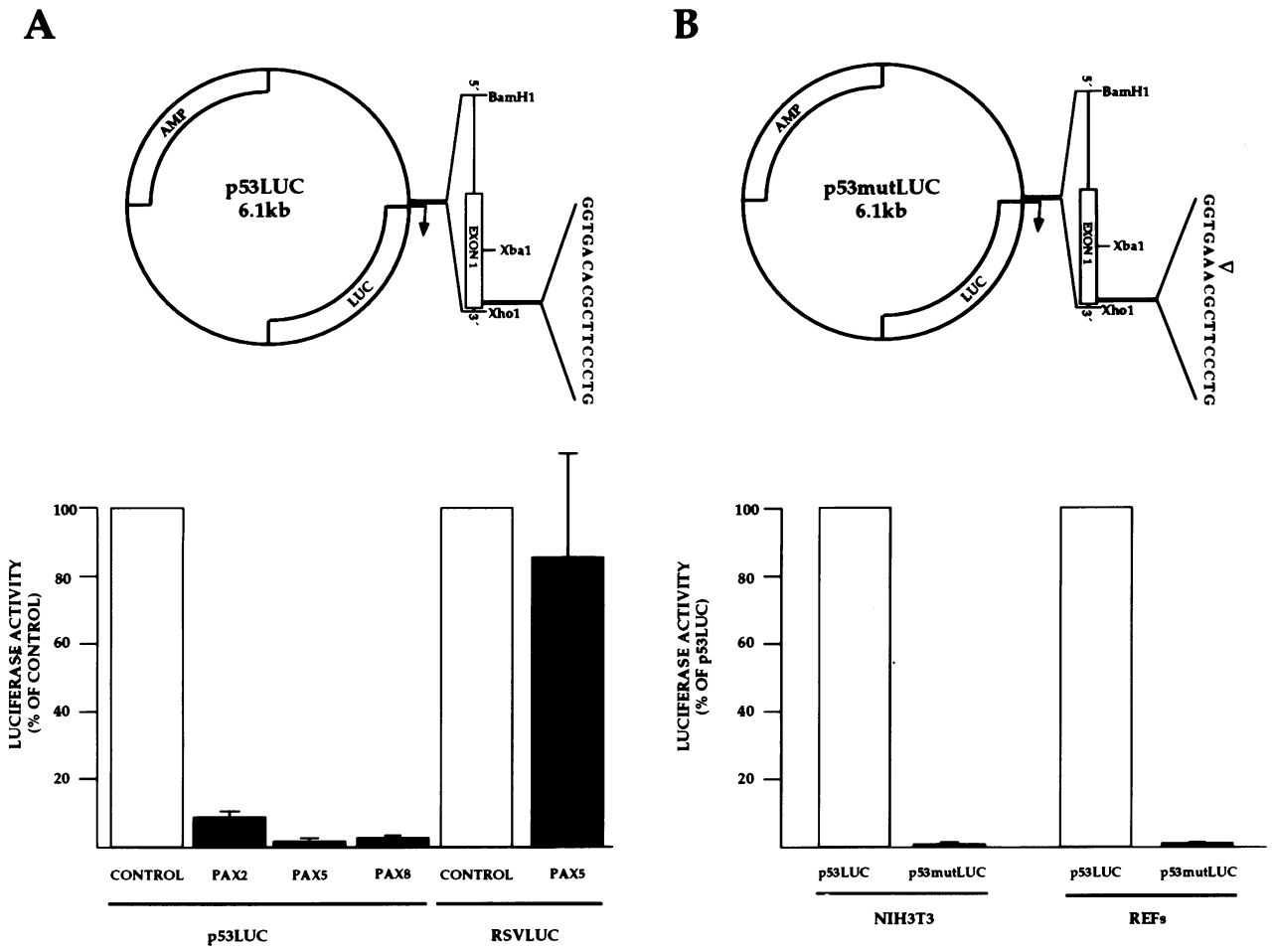

Fig. 5. (**A**) PAX proteins abolish activity of the human p53 promoter in transiently transfected primary rat embryo fibroblasts (mean ± SEM, $n = 5$ independent transfections). Upper panel, plasmid map of p53LUC showing the wild-type pentanucleotide of the PAX binding site. Lower panel, the cells were transfected with p53LUC (2 μg) and PAX expression plasmids (200 ng) or parental pCMV vector (CONTROL) by calcium phosphate precipitation. An unrelated promoter, RSVLUC (2 μg) was unaffected by PAX5 expression. (**B**) Point mutation abolishes activity of the human p53 promoter in transiently transfected NIH 3T3 and primary rat embryo fibroblasts (mean ± SEM, $n = 3$ independent transfections). Upper panel, plasmid map of p53mutLUC showing the mutated pentanucleotide of the PAX binding site. Lower panel, the cells were transfected with the reporter plasmids (2 μg) in parallel by calcium phosphate precipitation.

is determined and represents the 'stem cell' population for that organ or tissue. For proper development of that tissue, a precise combination of growth factors, transcription factors and other genes are required to guide the population along the proper pathway. In our model, we assume that a positive growth signal is presented to the cell. The positive growth signal activates *Pax* gene expression and the corresponding proteins cause a transcriptional inhibition of p53. This scenario continues until the signal is no longer sufficient to sustain *Pax* gene activation and the gene is transcriptionally inactivated. In the absence of PAX, the inhibition of p53 is alleviated and p53 is again able to be transcribed.

The functional implications of this model are that, during the initial phases, p53 is active and may help in determining the fate of the cell population. However, once the cell population is determined, a rapid growth of these (still undifferentiated) cells is required so that a large mass of cells is attained prior to differentiation. *Pax* genes, in part, mediate this rapid expansion. Once a 'critical mass' is obtained, PAX is no longer expressed and p53 is expressed.

The developing central nervous system is highly complex, requiring precise interactions between different cell types and fine control of cellular growth and differentiation rates so that the many different structures can be formed. PAX5 is expressed before differentiation in the cells which migrate from the ventricular zone to the intermediate zone of the neural tube (Asano and Gruss, 1992). The ventricular zone is characterized by its high mitotic rate prior to cellular differentiation. Upon cell differentiation, expression of PAX2, 5 and 8 ceases—unlike PAX3 which continues to be expressed in differentiated cells. Thus, inhibition of p53 in PAX5-expressing cells would agree with the highly proliferative and undifferentiated phenotype of these cells.

Recently, mutation of the murine *Pax5* gene has been described (Urbanék *et al.*, 1994). The homozygous mice display a number of phenotypes which include a complete block in early B cell differentiation. B cell differentiation proceeds along a well documented path from stem cells to the immunoglobulin-secreting plasma cells (for reviews, see Rolink and Melchers, 1991; Kantor and Herzenberg, 1993; Hagman and Grosschedl, 1994). PAX5 is expressed

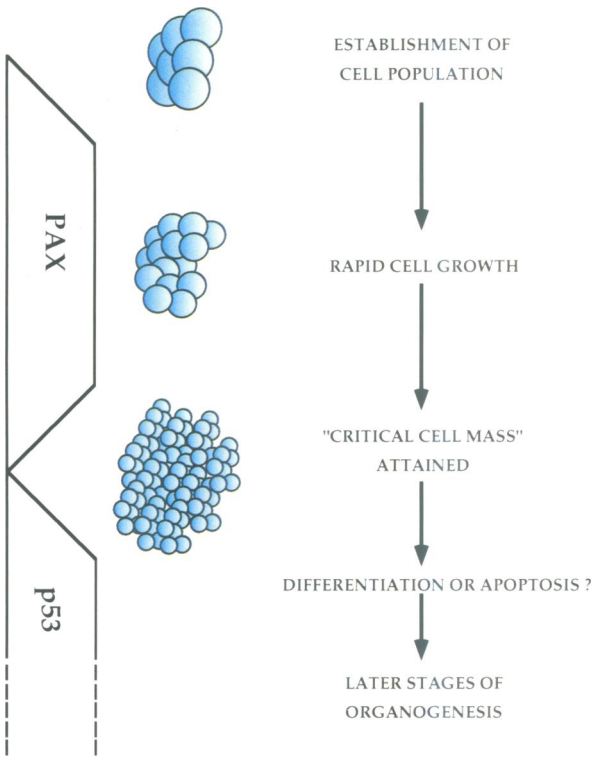

Fig. 6. Theoretical model for the involvement of PAX-mediated temporal down-regulation of the p53 gene during embryonic development.

during the early stages of B cell differentiation, i.e. up to the mature B cell stage and, in the later blast and plasma cell stages, PAX5 expression is no longer detectable (Barberis *et al.*, 1990; Adams *et al.*, 1992). However, in *Pax5* mutant mice, B cell differentiation is arrested in the early pro-B cell stage, with the cells displaying CD45 expression (which is normally lost during later differentiation stages) and the cells being morphologically different from the corresponding wild-type cells which undergo normal differentiation (Urbanék *et al.*, 1994). One feature of B cell differentiation is a selection at the mature B cell/blast stage prior to plasma cell genesis. This selective period determines which cells will progress through to plasma cells and which will undergo apoptosis (Rolink and Melchers, 1991). The mechanisms which underlie the apoptotic death of B cells are partly understood at present. *bcl-2* prevents apoptosis selectively in B cells (Vaux *et al.*, 1988; McDonnell *et al.*, 1989; Henderson *et al.*, 1991) and the *bax* gene encodes a protein which has opposite physiological functions (Miyashita and Reed, 1995). Both of these genes are potentially controlled by p53 (Miyashita *et al.*, 1994a,b; Miyashita and Reed, 1995). Therefore, it is conceivable that p53 is involved in B cell apoptosis. PAX5 expression would inhibit p53, the consequences of which would be an inhibition of BAX and continued expression of Bcl-2. Such a scenario would agree both with our observations and those of the *Pax5* mutant mice. We envisage that expression of PAX5 early in pro-B cell differentiation is required to inhibit p53 expression, leading to a rapid growth and partial differentiation phase during which complete differentiation is not permitted and, probably more importantly, the cellular decisions concerning apoptosis are prevented. Once PAX5 expression is reduced, p53 expression commences and the final stages of differentiation occur. In the absence of PAX5, the myriad of molecular mechanisms which it controls are deregulated and, in particular, p53 is active in the early phases during which it would otherwise not be active.

Implications for cancer

Inactivation of p53 is an extremely common event in human cancer (Levine *et al.*, 1991; Fults *et al.*, 1992). However, with the exception of functional inactivation of the p53 protein by either viral proteins (Ludlow, 1993) or the product of the *MDM2* gene (Momand *et al.*, 1992; Oliner *et al.*, 1992), all previously reported examples of p53 inactivation in human tumours have been due to mutations within the p53 gene or gross chromosomal rearrangements involving the p53 locus. The effect of mutating p53 has been studied widely and results in compromised sequence-specific DNA binding and transcriptional activity (Oren, 1992; Donehower and Bradley, 1993). Thus, the commonly encountered p53 mutations eliminate the normal tumour suppressor function of p53 by producing a functionally deficient protein. Our results indicate the existence of a novel mechanism for loss of p53 activity, namely the inhibition of p53 gene expression by PAX proteins. The constitutive expression of PAX5 or a related protein would thus bypass the need for p53 mutation to abrogate p53-mediated processes. Cancer is a multi-step process, which requires the cumulative effects altering both positive and negative regulators of cell proliferation and cell survival (Nowell, 1986; Vogelstein and Kinzler, 1993). Abrogation of p53 transcription by PAX may serve as an important factor in this multi-step process. In addition, it is conceivable that inappropriate PAX expression may contribute to tumorigenesis by activating specific target genes, as well as by inactivating other inhibitory genes in addition to p53.

Progression of a tumour to a higher state of malignancy is one of the most important aspects of cancer. Many tumours start as low malignancy neoplasms which, even after conventional treatment, progress to higher forms of malignancy. These often harbour metastatic potential which grossly alters patient prognosis. Our initial observation demonstrated that PAX5 is overexpressed in highly malignant glioblastoma multiform tumours, whereas tumours of lower malignancy (anaplastic astrocytomas, astrocytomas) did not express significant levels of PAX5 (Stuart *et al.*, 1995). We surmised that PAX5 may play a role in progression of the tumour, and its ability to inhibit p53 concurs with this. It is of note that if a mutant form of p53 was already present in the tumour cell and the promoter was not mutated, then PAX5 would also inhibit expression of this mutant p53. However, it is conceivable that p53 is only one of a set of PAX5 target genes whose activation or inhibition may combine to provide the cancer cell with a positive growth advantage. Thus, even in cells which have already lost wild-type p53 function owing to prior p53 gene alterations, the activation of PAX5 will probably have a net selective advantage.

Another tumour which may potentially relate to these observations is Wilms' tumour (nephroblastoma), a pediatric renal tumour affecting 1 in 10 000 children (for review, see Hastie, 1994). Phenotypically, Wilms' tumour is composed of undifferentiated mesenchymal stem cells

that have failed to undergo transformation to the epithelial components of the kidney. PAX2 and 8 are persistently expressed in Wilms' tumour, in contrast to their normal temporally regulated expression pattern in the undifferentiated developing kidney prior to the mesenchyme–epithelium transformation (Dressler and Douglass, 1992; Eccles *et al.*, 1992; Poleev *et al.*, 1992). The status of p53 has not been studied widely in Wilms' tumour, although it has been reported that *in vitro* the Wilms' tumour suppressor gene product WT1 can form a transcriptionally active complex with wild-type p53 and that p53 is mutated in a small subset of Wilms' tumours (Hastie, 1994). In view of the negative effects of PAX2 and 8 on p53, comparable with those of PAX5, it is tempting to speculate that the aberrant expression of these proteins in Wilms' tumour may also contribute to malignancy by compromising p53 function.

In conclusion, we have demonstrated a role for regulators of development in directing the cell cycle to allow the proper development of the embryo. In addition, reactivation of these pathways later in life may constitute an important novel mechanism for tumorigenesis in humans.

Materials and methods

Recombinant plasmids

PAX cDNAs were cloned into pCMV expression plasmids and have been described previously (Maulbecker and Gruss, 1993). RGC–CAT contains multiple copies of a p53 binding sequence located near the ribosomal gene cluster in front of the CAT gene (Kern *et al.*, 1991).

The p53LUC plasmid was made using PCR. A 4 kb *Eco*RI human genomic DNA fragment cloned into pBR322 was used as a template and PCR was performed with the following primers (from 5′ to 3′): sense, TTGAGCTCAGGATCCAGCTGAGAGCAAA; antisense, TTCTCGAGAATCCAGGGAAGCGTGTCACCGT.

PCR was performed in 10 mM Tris–HCl pH 8.85, 25 mM KCl, 5 mM $(NH_2)_2SO_4$, 2 mM $MgSO_4$ with 10 ng of DNA template, 0.1 μg of each primer and PWO polymerase (2.5 U) in a final volume of 100 μl. PCR was performed in an automated thermocycler (Biometra triothermoblock) and consisted of 30 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C. The PCR products were purified over spin columns (Qiagen), digested with *Bam*HI and *Xho*I and ligated into pBluescript and subsequently sequenced. Once the sequence was verified, the plasmid was digested with *Sac*I and *Xho*I and the insert subcloned into pGL-2 basic (Promega). Point mutation of the PAX binding site was performed as above using the antisense primer: 5′-TTCTCGAGAATCCAGGGAAGCGTTTCACCGT-3′. This point mutation introduces a novel *Xmn*I restriction site which was used to screen colonies. Those colonies which displayed the correct digestion pattern were sequenced. Once the sequence was verified, the plasmid was digested with *Sac*I and *Xho*I and the mutated insert was subcloned into pGL-2 basic.

Oligonucleotides

50mer oligonucleotides corresponding to all potential binding sites within the p53 promoter, exon 1 and 1.2 kb of the first intron as well as all point mutants used were synthesized on a BIOSEARCH 8700 DNA synthesizer and purified subsequently by HPLC. Double-stranded oligonucleotides were labelled with [^{32}P]dCTP (Amersham, UK).

Preparation of whole cell extracts and electrophoretic mobility shift assays

COS-7 cell extracts were prepared and EMSA performed as previously described (Chalepakis *et al.*, 1991). Briefly, COS-7 cells were transfected with 10 μg of the expression vectors [pCMV, pCMV(+)PAX1, pCMV(+)PAX2, pCMV(+)PAX3, pCMV(+)PAX5, pCMV(+)PAX6, pCMV(+)PAX8] using the calcium phosphate precipitation technique. Cells were harvested after 72 h and resuspended in 200 μl of extraction buffer [20 mM HEPES, 150 mM NaCl, 25% glycerol, 0.5 mM dithiothreitol (DTT) and protease inhibitors]. Cell suspensions were sonicated (Branson cell disruptor), the resulting mixture centrifuged and the supernatant frozen and stored at –80°C.

EMSAs were performed with the COS-7 extracts in a 15 μl volume containing 15 mM Tris–HCl (pH 7.5), 6.5% glycerol, 0.7 mM EDTA, 0.2 mM DTT, 90 mM KCl, 1 mg/ml bovine serum albumin, 100 ng of sonicated salmon sperm DNA and 15 fmol of labelled oligonucleotides. Protein–DNA complexes were allowed to form by incubation at room temperature for 30 min, and free DNA or protein–DNA complexes were resolved on 5% polyacrylamide gels in 0.5% TBE. Gels were dried and exposed to Kodak X-OMAT film at –80°C.

Cell culture and DNA transfection

All cell lines were cultured in DMEM (Gibco BRL) supplemented with 10% fetal calf serum (Boehringer Mannnheim). DNA transfections were performed in 60 mm tissue culture dishes using the calcium phosphate precipitation technique. Medium was changed 3 h before the addition of the precipitate. Twelve to 15 h after addition of the precipitate, the medium was removed, cells were washed three times with phosphate-buffered saline (PBS) and fresh medium added. Cells were harvested 48 h after addition of the precipitate.

Reporter plasmid assays

CAT activity was assayed according to Gorman *et al.* (1982). Cells were washed three times in PBS, harvested in 40 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, resuspended in 150 μl of 250 mM Tris–HCl pH 7.8 and lysed by freeze–thaw in liquid nitrogen at 37°C. Endogenous acetylases were inactivated by heating the lysed cells at 60°C for 7 min and the extracts cleared by centrifugation (12 000 *g*, 10 min at 4°C). Cell extracts were stored at –20°C until assayed. Fifty microlitres of extract were added to 105 μl of 250 mM Tris–HCl pH 7.8 and 0.125 μCi of [^{14}C]chloramphenicol and incubated at 37°C for 5 min. Twenty microlitres of 4 mM acetyl coenzyme A were added and the mixture incubated at 37°C for a further 30 min. The mixture was then extracted with 600 μl of ethyl acetate and centrifuged for 60 s. The ethyl acetate phase was removed, dried in a Speedvac and the pellet resuspended in 8 μl of ethyl acetate and dotted onto thin-layer chromatography (TLC) plates (Polygram SIL-G, Machery-Nagel). TLC was performed in a 95%:5% chloroform:methanol solution for ~60 min. TLC plates were exposed to Kodak X-OMAT film for 12 h at –80°C.

To assay luciferase activity, cells were washed three times in PBS, harvested in PBS, pelleted by centrifugation and directly lysed in 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane *N,N,N′,N′*-tetraacetic acid, 10% glycerol and 1% Triton X-100. Luciferase assays were performed in 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_3$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin and 530 μM ATP. Assays were performed either in a Turner designs model 20 luminometer or a Berthold Lumat LB 9501 luminometer.

Antibodies

Monoclonal mouse antibodies (Pab 240) against mutant p53 (Oncogene Science) were used at dilutions of 1:100. Detection antibodies: 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (FITC) conjugated to anti-digoxigenin sheep F(ab)′ fragments (Boehringer Mannheim), tetramethylrhodamine (TRITC) conjugated to goat antibody against rabbit IgG (Sigma) were used at 1:100 dilution. Cy3 conjugated to goat anti-mouse IgG [$F(ab)_2$ fragments] (Dianova) was used at 1:500 dilution.

In situ hybridization–immunohistochemistry

Sections (13 μm) were cut with a cryostat at –20°C and transferred onto slides subbed with gelatine and chromalum. Sections were dried at 37°C, fixed for 20 min with 4% paraformaldehyde and rinsed in PBS. Sections were pre-hybridized (200 μl per slide) with 5× SSC, 5× Denhardt's, 50% deionized formamide, 250 μg/ml of yeast tRNA, 250 μg/ml of denatured salmon sperm DNA and 4 mM EDTA, for 2–3 h in a humid 45°C chamber, and washed at room temperature for 2 min in 70, 90 and 100% ethanol. Digoxigenin-labelled RNA probes were synthesized, using T7 or T3 polymerase according to the supplier's directions (Boehringer Mannheim) from the linearized plasmid cDNA clone described above. Sections were incubated as above (with probe) in pre-hybridization buffer lacking salmon sperm DNA (2.5 ng/μl), and washed twice for 15 min with 2× SSC, 15 min with 0.2× SSC and twice for 15 min with 0.1× SSC at 45°C. For double-labelling fluorescence, cells were incubated with primary antibodies for 2 h at room temperature, rinsed, and both secondary antibodies applied for 2 h. Controls were: (i) using sense probe; (ii) omitting the probe, first antibody, second antibody or all of the above. Immunofluorescence stained preparations

were viewed with a Zeiss Axiophot photomicroscope with phase contrast corresponding to the appropriate magnification.

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