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The oncogenic potential of Pax genes

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Our results demonstrate that murine paired domain-containing genes (Pax) can promote oncogenesis in tissue culture cells and in mice, and should thus be classified as a novel group of proto-oncogenes. The induction of tumor formation in mice was dependent on a functional paired domain, but did not require the presence of a homeodomain. Consequently, not only the Pax-3 and Pax-6 proteins, which in addition to paired domains contain intact homeodomains,but also Pax-2 and Pax-8, containing only residual homeodomains, and Pax-1, completely lacking a homeodomain, were able to induce transformation of cell cultures and tumor formation in mice. The oncogenic potential of the Pax proteins is dependent on the DNA binding function of the paired motif, as the Un−Pax-1 protein, which carries a point mutation in this domain that impairs DNA binding, is also defective in tumor formation. Therefore, the Pax gene products are not only involved in controlling embryogenesis, but they can, if deregulated, also induce tumorigenesis.

Key words: Pax/proto-oncogenes/tumorigenesis

Introduction

Homeobox-containing proteins play an important role in the development of multicellular organisms. Transcriptional regulation by the homeobox proteins is thought to coordinate the precise spatial and temporal sequence of growth, and differentiation in the developing embryo. A role of homeodomain-containing proteins in the differentiation processes in the hematopoietic system and in the appearance of myeloid leukemia on upregulation of the Hox-2.4 protein point towards a link between tumorigenesis and development (Blatt *et al.*, 1988; Kongsuwan and Adams, 1989; Blatt, 1990; Aberdam *et al.*, 1991). In two cases acute leukaemias have been correlated with abnormal regulation of human homeodomain-containing proteins (*PBX-1* and *HOX-11*) (Kamps *et al.*, 1990; Nourse *et al.*, 1990; Rabbitts, 1991 for review). Recently, the paired-type homeobox-containing gene *Pem* has been described as an 'oncofetal' gene because it is expressed in fetal and tumor cells (Sasaki *et al.*, 1991). These findings provide circumstantial evidence that some homeodomain-encoding genes that are involved in regulating embryogenesis and hematopoiesis can also promote oncogenesis.

Development and oncogenesis thus appear to be conversely related in that these processes involve either the regulation or deregulation of growth. We have therefore addressed the question of whether another class of abnormally regulated developmental control genes can have an oncogenic function.

To this end we have studied the Pax class of developmental control genes. Three members of this gene family (*Pax-1, Pax-3* and *Pax-6*) have been linked with developmental mutants in the mouse (Balling *et al.*, 1988; Epstein *et al.*, 1991; Hill *et al.*, 1991) and, in two cases, with the equivalent human syndromes, Waardenburg/*PAX-3* (Baldwin *et al.*, 1992; Tassabehji *et al.*, 1992) and Aniridia/*PAX-6* (Ton *et al.*, 1991). Pax-2 and Pax-8 proteins have been shown to be expressed at high levels in Wilms tumors, which suggests a link between the developmental control gene and tumorigenesis (Dressler and Douglas, 1992; Eccles *et al.*, 1992; Poleev *et al.*, 1992).

While only some Pax genes encode proteins that contain a complete paired-type homeodomain (Deutsch and Gruss, 1991; Gruss and Walther, 1992 for review), all encode a DNA binding motif termed the paired domain (Bopp *et al.*, 1986; Walther *et al.*, 1991). Another conserved sequence that is present in most Pax genes is the octapeptide, the function of which is not known (Burri *et al.*, 1989; Walther *et al.*, 1991). In order to determine the oncogenic potential of Pax genes and to identify the qualitative contributions of domains, which might be required for transformation, we studied Pax genes that encode both the paired domain and a complete homeodomain (*Pax-3* and *Pax-6*), as well as Pax genes that encode only a residual homeodomain (*Pax-2* and *Pax-8*), or no homeodomain (*Pax-1*) (Figure 1). *Pax-6*, in addition, lacks the octapeptide.

To correlate the DNA binding function of the paired domain (Hoey and Levine, 1988; Treisman *et al.*, 1991) with a potential role in transformation we also included the Un protein in our study. This protein is identical to the Pax-1 protein except for a point mutation in the paired motif that reduces DNA binding (Chalepakis *et al.*, 1991). This mutation is thought to be the cause of the phenotype in the undulated mouse mutant (Balling *et al.*, 1988).

We report here that paired box proteins are capable of inducing transformation both *in vivo* and *in vitro*.

Results and discussion

Experimental strategy

It has been shown that a threshold exists below which oncogene expression has no detectable effect on transformation (see Hunter, 1991 for review). We intended to overcome such a potential threshold by constitutively overexpressing Pax proteins under the control of the cytomegalovirus (CMV) promoter/enhancer, thus raising Pax protein levels above the low endogenous background seen in some cell lines (i.e. differentiated EC cell lines).

In order to test whether Pax proteins can transform non-expressing recipient tissue culture cells, it was necessary to express Pax cDNAs in these cells. To this end, cDNAs for

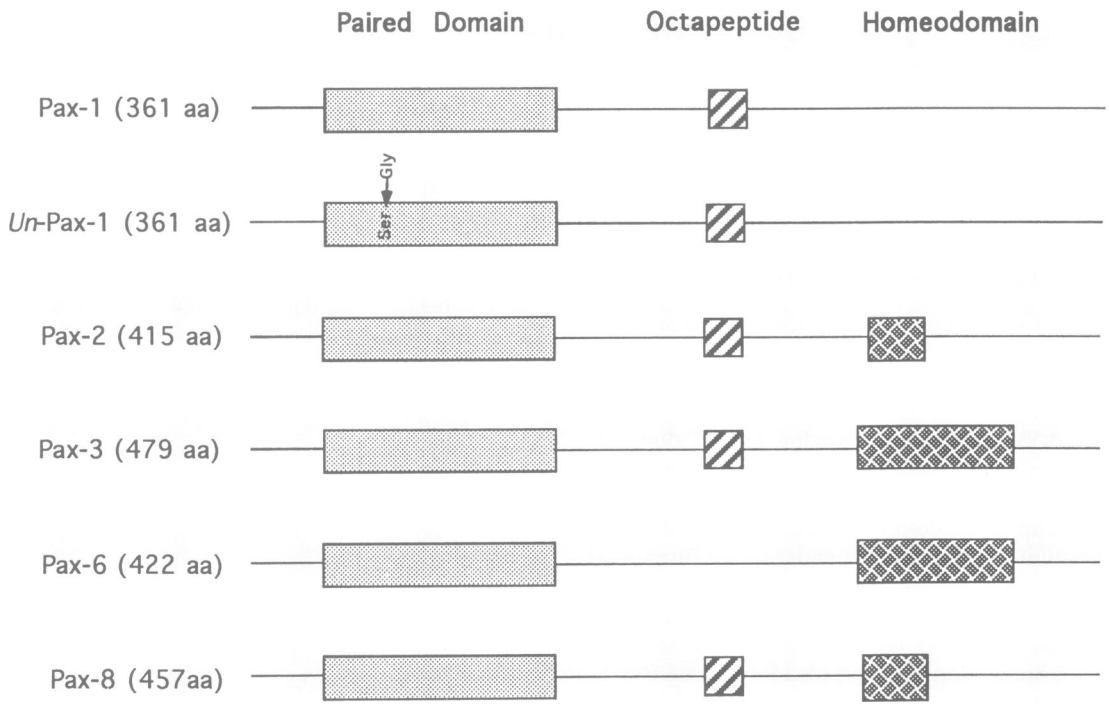

Fig. 1. Schematic structure of the Pax proteins. The lengths of the boxes and lines are not drawn to scale, but serve to illustrate the appropriate positions of important structural motifs within the proteins. The sizes of the Pax proteins in amino acids are indicated. The conserved paired domain is present in all Pax proteins. The point mutation of G to A in the paired domain of the gene encoding the Un protein is indicated by the resulting Gly to Ser change. The conserved octapeptide is present in all Pax proteins except Pax-6. Pax-1 is the only Pax protein known that completely lacks a homeodomain. Pax-3 and Pax-6 contain complete paired-type homeodomains, in addition to the paired domain. The two DNA binding motifs are separated by at least 100 amino acids. The Pax-2 and Pax-8 proteins contain only 23 amino acids of the first α helix of the homeodomain.

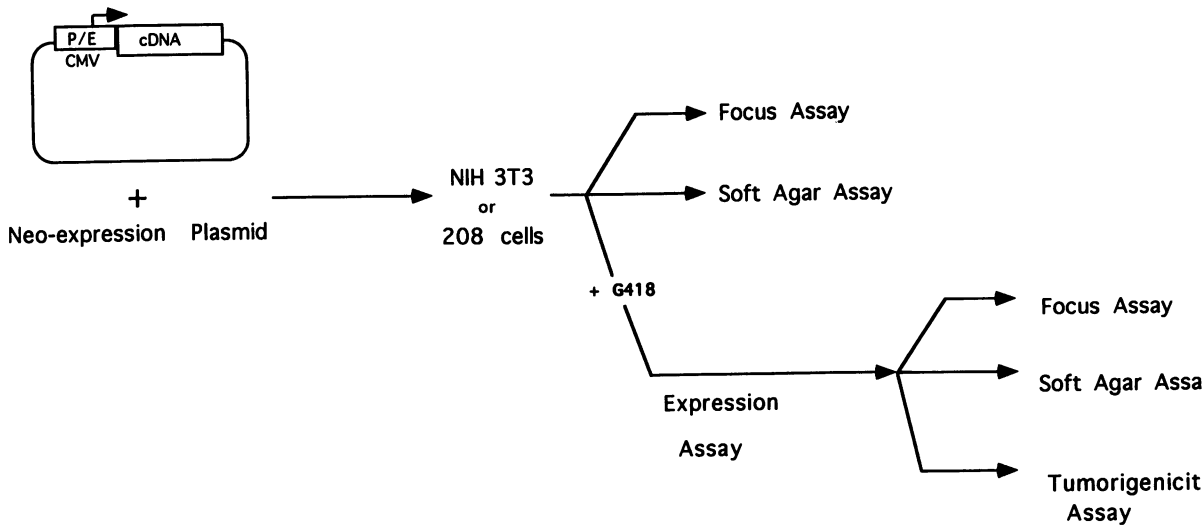

Fig. 2. Experimental scheme for the transformation assay. Pax cDNAs were inserted into the multiple cloning site of pCMV5. pCMV5 contains the promoter/enhancer (P/E) region of the cytomegalovirus followed by a multiple cloning site, an intron and polyadenylation signals (Anderson *et al.*, 1989). The expression construct was co-transfected with pGKneo, conferring resistance to neomycin, into either NIH 3T3 or 208 cells. Each transfection was split 1:3 after 24 h. One plate was split 1:2 and left for 2–4 weeks depending on the onset of focus formation. Upon the appearance of foci, the cells were fixed and stained, and the foci counted. A second plate of cells was seeded into either 0.3, 0.6, 0.9 or 1.2% soft agar. The last third of the transfected cells was selected for DNA uptake. Colonies of morphologically transformed cells were picked and propagated. These cell clones were expanded and used for expression analysis and for *in vitro* (focus assay, soft agar assay, cell mixing experiment) and *in vivo* (tumorigenicity in nude mice) transformation assays.

Pax-1, Un, Pax-2, Pax-3, Pax-6 and *Pax-8* were subcloned into a pCMV expression vector (Anderson *et al.*, 1989) (Figure 2). The CMV promoter/enhancer strongly expresses the cDNA inserted into the multiple cloning site of this vector. These constructs were co-transfected into 208 and NIH 3T3 cells together with pGKNeo, which carries a selectable marker (Soriano *et al.*, 1991). 208 cells were derived from a Rat-1 cell subclone, isolated for its lack of spontaneous transformation when grown in tissue culture (R.Müller, personal communication). The NIH 3T3 cells were a gift from F.Cuzin. Two-thirds of each transfected cell population were grown for 2–4 weeks and then one-

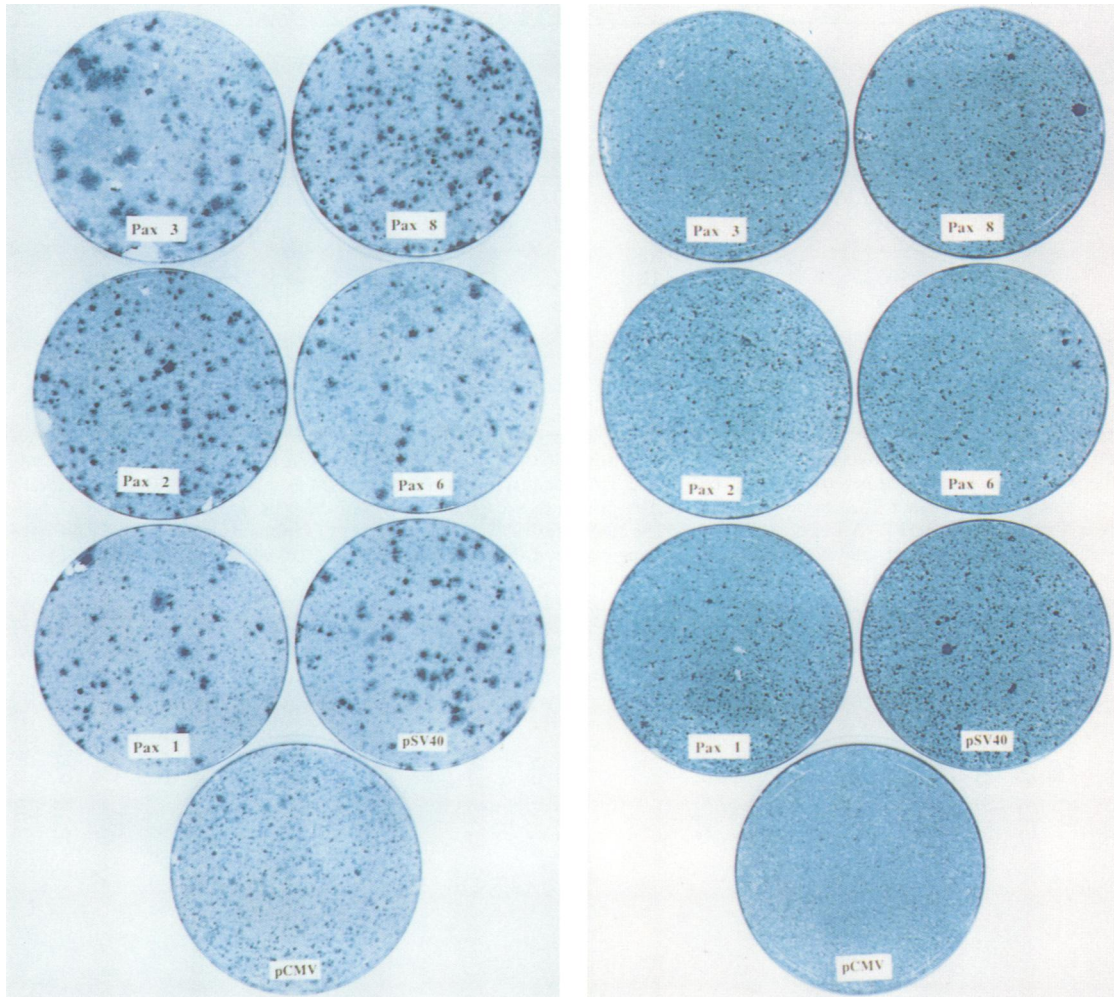

Fig. 3. Focus assay for the ability to overcome contact inhibition. This assay was performed twice in untreated cells after transfection and twice by mixing established cell clones with untreated cells. The left and right panels show NIH 3T3 and 208 cells transfected with Pax protein-expressing constructs, respectively. Cells that have taken up the transforming DNA are able to overgrow non-transformed cells, resulting in dark-staining cell foci. Depending on transfection efficiencies, the number of foci obtained varies in the plates shown (see Table I). On the bottom of each panel, control cells that harbor only the pCMV construct are shown. No foci were visible in the 208 cells, whereas the NIH 3T3 cells displayed a significant background. pSV40-harboring cells exhibited strong focus formation, the onset of which occurred about 1 week earlier than for the Pax-transformed cells.

third was stained with methylene blue for focus formation (Figure 3). The NIH 3T3 cells provided higher levels of spontaneous foci in this assay when compared with 208 cells (Table I). The second third of the transfected cells was tested for growth in soft agar. Growth properties of these unselected cells were similar to the results obtained when selected cell clones of the respective Pax construct were tested (see soft agar assay and Table I). The remaining third of the cells was selected with G418 for DNA uptake and morphologically transformed cell clones were isolated (Figure 2). Two independent cell clones for each expression construct as well as transfection pools were assayed for the expression of the respective Pax proteins. In cases in which antibodies were available expression was examined by Western blot analysis (Pax-1, Pax-2, Pax-3, Pax-6 and Pax-8) (Figure 4). The Pax-2 antibody cross-reacted with Pax-8 and allowed the detection of *Pax-8* in the respective cell clones (data not shown). The endogenous levels of Pax proteins are below the level of detection in the cell lines used. Consequently, cells transfected with the insertless pCMV vector alone (lanes labelled 208 CMV and 3T3 CMV) gave no detectable signals in Western blots using 50 μg of total protein from cell extracts in each lane (Figure 4). All Pax cell clones expressed the respective Pax proteins at levels that allowed their detection in 50 μg total cell extract on Western blots. This compares well with the low endogenous expression of Pax proteins, which in expressing cells and tissues can normally be detected only upon prior enrichment by immunoprecipitation (Chalepakis *et al.*, 1991; Goulding *et al.*, 1991).

For Pax-1 and Un, gel retardation analysis was performed using conditions described previously for Pax proteins (Figure 4) (Chalepakis *et al.*, 1991). The Pax-1 and Un proteins were expressed at similar levels, as demonstrated by Western blot analysis (Figure 4A). Un, as expected, demonstrated greatly reduced binding to the target DNA in gel retardation assays (Figure 4B). Cell extracts from Pax-2, Pax-3 and Pax-8 stably transfected cells exhibited binding to the target oligonucleotide PRS-4 (PRS-9 was used for Pax-6-transfected cells) (Chalepakis *et al.*, 1991), confirming that the respective functional Pax proteins were expressed (data not shown).

Table I. Transformation of NIH3T3 and 208 cells by Pax expression plasmids

Transfected DNA	Soft agar assay						Focus assay		Tumorigenicity
	No. of colonies/150 ng DNA						No. of colonies/300 ng DNA		No. tumors in nude mice/ no. of injections)
	0.6%		0.9%		1.2%				
	208	3T3	208	3T3	208	3T3	208	3T3	208
pCMV	0	3	0	0	0	0	0	5	0/5
pSV	>100	>100	73	>100	69	87	67	71	6/6
Pax-1	>100	>100	86	>100	11	63	38	44	6/6
Un	0	9	0	0	0	0	0	0	1/6
Pax-2	>100	>100	26	85	46	103	46	79	6/6
Pax-3	>100	>100	83	56	17	5	59	81	6/6
Pax-6	>100	>100	19	80	8	12	26	43	4/6
Pax-8	>100	>100	39	>100	3	94	31	107	6/6

Transformation of 208 cells with Pax proteins. The column on the left lists the DNAs that were introduced into the cells. pCMV indicates cells that harbor only the pCMV construct as the negative control, pSV is the T antigen expression construct used as a positive control and the various pCMV−Pax expression constructs are listed by the name of the Pax protein they express. Growth of the cells was tested in 0.3, 0.6, 0.9 and 1.2% soft agar. Cell colonies were counted 2−3 weeks after plating. The results for 0.3% are not listed, as even the negative control cells were able to grow. The average of two independent transfections of each cell type was used, in which the number of colonies obtained represents 1×10^4 cells transfected with 150 ng expression plasmid. In all cases, 208 cells were less able to grow in high soft agar concentrations than NIH 3T3 cells. The colony numbers obtained in the focus formation assay, testing the outgrowth from a cell monolayer, is presented in the next column. Colonies obtained represent $\sim 1 \times 10^4$ cells transfected with 300 ng of expression plasmid (Figure 3). These numbers were in agreement with a cell mixing experiment, measuring focus formation upon mixing untransformed 208 or NIH 3T3 cells with the transfected cell colonies (data not shown). The last column indicates the number of injections that resulted in tumor formation and the number of nude mice injected.

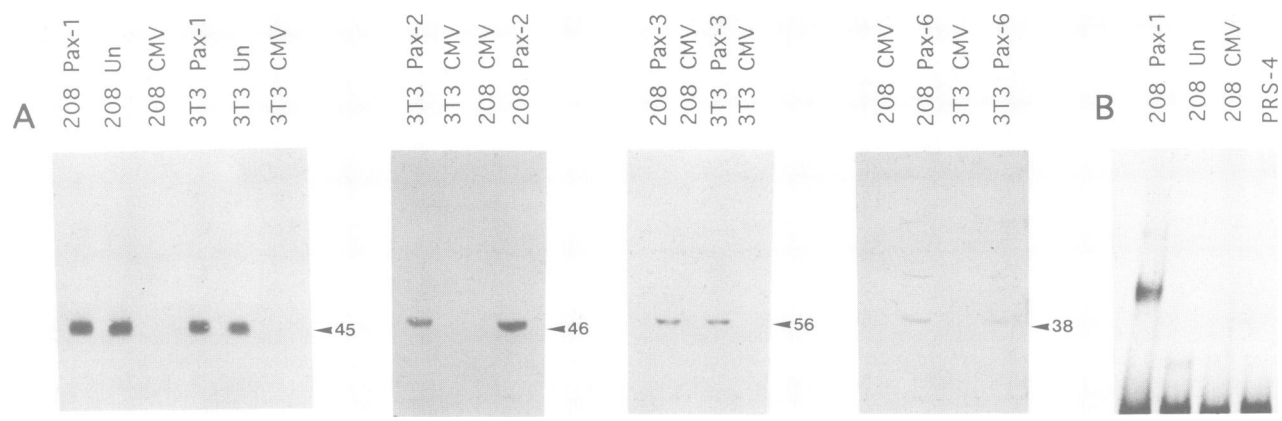

Fig. 4. (A). Western blots of Pax-expressing cell extracts. The molecular weights of the Pax proteins were estimated by comparison with the rainbow protein molecular weight marker (Amersham). The sizes of the proteins are given in kDa. Both 208 and NIH 3T3 cell extracts contain similar amounts of the Pax-1, Pax-2, Pax-3 and Pax-6 proteins per 50 μg total protein. The Pax-1 Western blot (far left), shows that the Un and Pax-1 proteins are expressed at about equal levels. In all cases, the Western blots show that the insertless pCMV-harboring control cells (208 CMV and 3T3 CMV) express much lower or undetectable amounts of the respective proteins. **(B).** Gel retardation experiments verify that the Pax proteins expressed can bind to DNA. 10 μg of total protein from whole cell extracts expressing Pax-1 or Un were incubated with end-labelled PRS-4 (PRS = paired domain recognition site) oligonucleotide under binding conditions described for Pax proteins (Chalepakis *et al.*, 1991). The nucleotide sequence of this oligonucleotide is: TCGACTAGTCACTGGGCTCACCGTTCCGCTCTAGATATC. Extracts from cells harboring insertless pCMV alone or from Un-expressing cells, did not give rise to a shifted DNA−protein complex.

Soft agar and focus assays

As cell growth in increasing concentrations of soft agar can be correlated with the likelihood of tumor formation in permissive animals (Fidler *et al.*, 1991), Pax-expressing clones were tested in soft agar assays of varying stringency. Cells containing the insertless pCMV expression vector showed little growth in 0.3% soft agar and no growth in higher soft agar concentrations. Pax-1-, Pax-2-, Pax-3-, Pax-6- and Pax-8-expressing cells were able to grow at concentrations ranging from 0.3% to 1.2% soft agar (Table I). *Un*−Pax-1 protein was unable to fully transform these cells, as shown by the lack of anchorage-independent growth in higher soft agar concentrations (Table I). Qualitatively similar results were obtained when the respective Pax transfections were seeded prior to selection into soft agar. Growth of cells suspended in this semi-solid medium indicates that Pax proteins confer the ability of anchorage-independent growth.

NIH 3T3 and 208 cells that had been co-transfected with pGKneo and pCMV, and selected for G418 resistance, were tested in a control transformation experiment to exclude effects due to growth in the selection medium. In this experiment non-transfected cells were mixed with transfected cells in the ratio 1000 to 1. Cells were stained with methylene blue after the appearance of foci. Foci appeared only in those experiments in which Pax transfectants were mixed with non-transfected cells and not when cells transfected only with pCMV and pGKneo were used (data not shown), thus

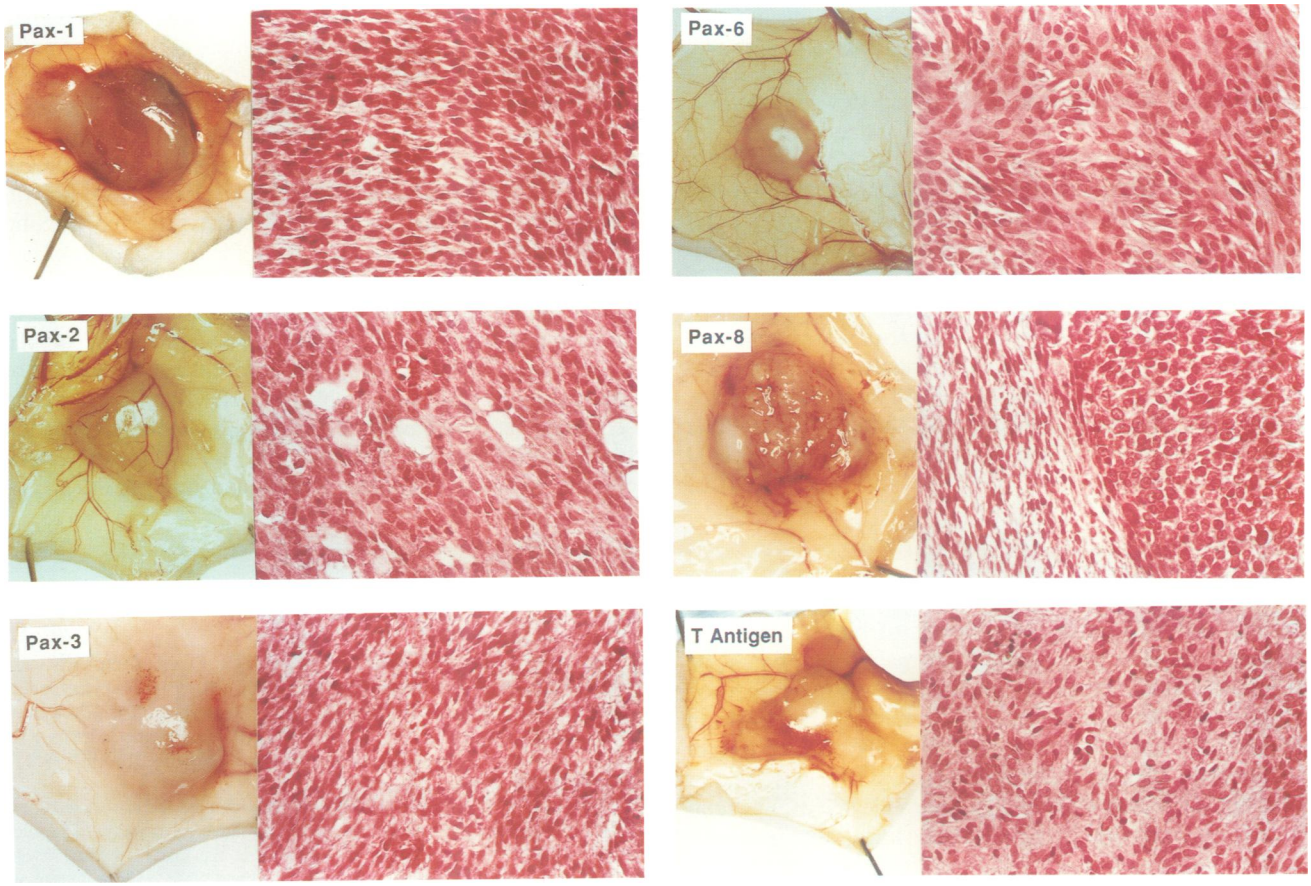

Fig. 5. Photographs of the tumors induced through Pax transformation. Tumors were extracted, photographed and embedded according to standard *in situ* hybridization protocols (Goulding *et al.*, 1991). Pax-1, Pax-2, Pax-3, Pax-6, Pax-8 and T antigen 208 cell tumors are shown on the left with hematoxylin-stained sections of the tumor on the right. The histology photographs were taken at a ×250 magnification. When compared with the cuboidal T-antigen tumor, the cells in the Pax tumors are spindle-shaped. The tumors are well vascularized, as can be seen by the large blood vessels and the surrounding blood islands, as well as the reddish color of the tissue. Tumors induced by the *Hox-2.4* gene, in contrast, were described to have a pale appearance due to their failure to induce angiogenesis (Blatt *et al.*, 1988). The Pax tumors show abundant extracellular matrix production, as evident in the Pax-8 tumor section. The Pax-8 tumor was dimorphic, with regions of solid round cell foci as well as regions of spindle-shaped cells with abundant extracellular matrix. All Pax tumors were solid and encapsidated.

supporting the *in vitro* transformation data obtained with unselected transfected cells.

The appearance of strongly stained foci in the methylene blue assay correlated with growth in the soft agar assay for 208 and NIH 3T3 cells transfected with constructs expressing Pax proteins that contained functional paired domains (Figure 1 and Table I). Cells expressing the Pax protein were capable of overcoming contact inhibition and hence overgrew the cell monolayer, visible as dark staining foci of cells similar to those shown in Figure 3. The *in vitro* transformation exhibited in both soft agar and focus assays was qualitatively identical when either newly transfected cells or G418 resistant cell clones were used. Other developmental control genes that encode different classes of transcriptional activators, such as octamer proteins (Oct-4) and zinc-finger proteins (Zfp-2) (Chowdhury *et al.*, 1988; Schöler *et al.*, 1990) showed no significant effect on focus formation and growth in soft agar in either of the *in vitro* transformation assays (data not shown). The appearance of Pax-induced foci was delayed ~1 week when compared with 208 and NIH 3T3 cells that had been transfected with the SV40 large T antigen-expressing vector (pSV), used as a positive control in the transformation assays. This suggests that the transforming ability of the Pax proteins is weaker than that of the viral oncogene. However, the number of foci obtained in four independent transfections was roughly similar for both cell types expressing the Pax proteins and the T antigen.

Tumorigenicity assay

208 cells harboring either expression constructs for the Pax proteins or the pSV and pCMV controls were injected subcutaneously into 4 week-old male nude mice. After three months the mice were sacrificed and scored as tumor-free if no tumors had appeared. In the case of earlier tumor growth, the animals were sacrificed once a tumor of ~1 cm in diameter had appeared. The cell number injected was maintained at $1-5 \times 10^5$ cells per injection. In one out of six mice injected with the *Un*-expressing cells a small non-vascularized lump of cells appeared. In repeated injections with the same cell clone this result was not reproduced (Table I). Tumor formation was observed as early as 10 days post-injection for Pax-2 and pSV-T antigen-expressing cells. In similar studies with oncogenes such as *ras*, tumors appeared 7–10 days after injection (Land *et al.*, 1983). The injection of Pax-1-, Pax-3-, Pax-6- and Pax-8-expressing cells resulted in the growth with a latency of 2–6 weeks. T antigen- and Pax-protein-induced tumors were well vascularized and firm (Figure 5). Tumor cells that were transferred back into tissue culture exhibited growth rates and growth characteristics (growth in soft agar, growth in multiple layers) and cell

morphologies similar to the cells that had been injected into the animal. These tumor cells were established as stable cell lines.

To evaluate the cell morphology the tumors were prepared, embedded and sectioned following standard *in situ* hybridization protocols (Goulding *et al.*, 1991). Morphologically, the pax-induced tumors resembled spindle cell sarcomas (Figure 5). Infiltration into surrounding muscle and nerve tissues as well as blood vessel walls was observed. Furthermore, Pax tumor cells showed high mitotic activity and atypical mitosis (A.Aguzzi, personal communication). In contrast, the T antigen-induced tumors were round cell sarcomas that appeared less differentiated than the Pax-induced spindle cell sarcomas. Based on these observations, we suggest that spindle cell sarcomas, particularly those that occur early in development, should be tested for elevated Pax gene expression.

Conclusions

We have demonstrated that Pax gene products have transforming potential. The tumors formed in nude mice are well vascularized, suggesting recruitment of blood vessels by angiogenesis. The next step to full malignancy would be metastasis, which we did not observe in Pax tumors induced by subcutaneous injection. Even at tumor sizes of 1 cm diameter the animals appeared healthy (no loss of weight or mobility). No secondary tumors could be observed at locations other than directly adjacent to the site of injection. We did not, however, keep the animals longer than 3 months or allow tumor sizes to exceed 1 cm diameter, and therefore cannot exclude the possibility that metastasis could occur if malignancy progressed through a longer incubation. A strong correlation between anchorage-independent growth and metastatic behaviour has previously been observed (Fidler *et al.*, 1991), suggesting that the Pax genes may well be capable of inducing metastasis, since growth in up to 1.2% soft agar was observed. According to these criteria, Pax genes can be classified as proto-oncogenes that upon overexpression, induce focus formation, anchorage-independent growth and vascularized tumors.

Our results demonstrate that the Pax paired domain, in the absence of either the paired homeobox or the octapeptide, is required for transformation. Because the transforming potential of the paired domain appears to be dependent on its DNA binding capacity, the question arises as to what the targets of its transcriptional regulation could be. Prior unpublished *in situ* hybridization studies for at least some of the Pax genes used in the transformation assay correlate Pax gene expression with mitotic activity in developing tissues (Deutsch and Gruss, 1991; Gruss and Walther, 1992). These observations support the notion that Pax genes might be involved in growth regulation. Cell cycle proteins, such as string or cdc2 whose steady state levels are growth regulated, would be good candidates for common targets of developmental control genes (O'Farrell *et al.*, 1989). The loss of function in the *Un* mutant might then result in the deficient upregulation of such mitotic regulators, in the case of *Pax-1−Un* leading to deficient growth in embryogenesis (Balling *et al.*, 1988). Due to chromosomal translocations, as often seen in acute leukemias (Rabbitts, 1991), or as in our experiments due to the constitutive strong CMV promoter, the homeodomain or paired-box proteins are overexpressed (C.Maulbecker and P.Gruss, in preparation). The overexpression of these developmental control genes may affect common target genes and promote abberrant growth. In this respect it would be interesting to determine whether the transforming activity of Pax gene products is due to deregulation of another proto-oncogene. Initial experiments (G.Chalepakis and P.Gruss, in preparation) show an upregulation of c-*fos* in transfected cells. In development the growth promoting effect may be combined with a growth restricting effect through the downregulation of the respective Pax gene expression in a specific differentiating tissue. Both development and tumorigenesis seem intricately linked in terms of their molecular basis. A clearer understanding of the mechanisms and the targets of transcriptional regulation of the paired domain may help us to elucidate the common controls that coordinate cell proliferation and differentiation throughout embryonic development, and also the regulatory events promoting aberrant growth and tumorigenesis.

Materials and methods

Cell lines and mice

The NIH 3T3 cells are a subclone provided by F.Cuzin. These cells were maintained at low passage numbers in DMEM (Biochrome) supplemented with 5% new-born calf serum (Boehringer Mannheim). The 208 F cells are a subclone of Rat-1 cells maintained in DMEM supplemented with 10% fetal calf serum. These cells were originally isolated by R.Müller (IMT, Marburg, Germany).

Three week old athymic (nude) male NMRI mice were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). The mice were injected subcutaneously in the flank at the age of 4 weeks with $1-5 \times 10^5$ cells. The cells were trypsinized and washed twice with phosphate-buffered saline prior to injection to exclude stimulating effects from the serum. The animals were examined for tumor formation for up to 3 months.

Transformation assays

Pax-3 and Pax-6 cDNAs were inserted into the multiple cloning site of pCMV5 (Anderson *et al.*, 1989). Pax-1, Pax-2, Pax-8 and *Un*−Pax-1 were subcloned into pEVRF2 (Mathias *et al.*, 1989), which also expresses the inserted cDNA from the CMV promoter/enhancer region. 2 μg of the pCMV-Pax expression plasmid, together with 1 μg pGKneo (Soriano *et al.*, 1991) and 7 μg carrier DNA, were transfected on 70% confluent cell monolayers in a 100 mm tissue culture dish using the calcium phosphate method, with modifications (Weber and Schaffner, 1984). The transfected plates were split 1:3 after 24 h. One plate was left for 2−4 weeks, depending on the onset of focus formation. Thereafter the cells were fixed with a few drops of glutaraldehyde (Sigma) and stained with methylene blue (1% in water) (Sigma). The tissue culture plates were rinsed with water and the foci counted.

One-third of the cells was seeded into either 0.3, 0.6, 0.9 or 1.2% soft agar as previously described (Fidler *et al.*, 1991). The remaining third was selected for DNA uptake by the addition of G418 (Gibco) 24 h post-shock. The 208 cells received 0.4 mg/ml and the NIH 3T3 cells 0.6 mg/ml of G418. Morphologically transformed foci were picked and propagated thereafter. In some cases, pools of G418-resistant cell clones were also used. These cell isolates were expanded by continuous incubation in the selection medium. Thereafter the cells were tested in soft agar assays, mixed with untransfected cells and assayed for methylene blue stained foci and tested for *in vivo* transformation in nude mice.

Expression analysis

The Western blot analysis for Pax gene expression was performed with whole cell extracts, which were prepared essentially as previously described (Chalepakis *et al.*, 1991). The protein concentration was determined and 10 μg aliquots of the extracts were stored at −70°C. 50 μg total protein of cell extracts were separated on 12.5% SDS−polyacrylamide gels and transferred onto Immobilon-P membrane by semi-dry electric transfer. The membrane was blocked in 5% dry milk powder−PBS, incubated overnight with a 1:200 dilution of the respective Pax antiserum and developed with the peroxidase−diaminobenzidine reaction.

10 μg of total protein from the same extracts were used for gel retardation

analysis with an end-labelled oligonucleotide, as previously described for the Pax-1 protein (Chalepakis *et al.*, 1991).

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