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Pax-QNR/Pax-6, a Paired- and Homeobox-Containing Protein, Recognizes Ets Binding Sites and Can Alter the Transactivating Properties of Ets Transcription Factors

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We have previously isolated a cDNA clone encoding a protein with a paired- and homeodomain from MC29-transformed quail neuroretina cells that we have termed *Pax-QNR*. *Pax-QNR* is homologous to the murine *Pax-6*, which is mutated in the autosomal dominant mutation *small eye (Sey)* of the mouse and *aniridia* in man. The 46 kDa Pax-QNR protein binds specifically to the e5 DNA recognition sequence present upstream of the *Drosophila even-skipped* gene. The Pax-QNR paired and homeobox domains expressed separately in bacteria are both able to recognize this sequence. The core sequence recognized by the paired domain of *Pax* genes is TTCC (GGAA), and this sequence is also present in the core recognition site bound specifically by Ets family-encoded proteins. Ets proteins are a family of transcription factors sharing a highly conserved 85 amino acid DNA binding domain. In this article we demonstrate that Pax-QNR/Pax-6 expressed in reticulocyte lysate is able to specifically recognize several Ets binding sites. In addition, we found that the transactivation mediated by the $p68^{c\text{-}ets\text{-}1}$ or $p55^{erg}$ through the Polyomavirus enhancer sequence is specifically inhibited by the $p46kDa^{Pax\text{-}QNR}$ in transient transfection assay.

HOMEOBOX-containing genes encoding developmentally regulated transcription factors play an important role in the development of multicellular organisms. Transcriptional regulation by homeobox proteins is thought to coordinate the precise spatial and temporal sequence of growth and differentiation, and previous studies of *Drosophila* homeobox-containing genes have shown that these genes are involved in the process of pattern formation (see Akam, 1987 and Ingham, 1988 for review). Acute leukaemias have been correlated with abnormal regulation of homeodomain-containing proteins (see Rabbitts, 1991 for review), and immortalized murine fibroblasts can be transformed by overexpression of homeobox genes (Maulbecker and Gruss, 1993a). The homeobox encodes a conserved DNA binding domain (Treisman et al., 1989) containing a helix–turn–helix motif (Kissinger et al., 1990).

Another class of developmental control genes (*Pax* genes) has recently been found to transform immortalized murine fibroblasts when overexpressed (Maulbecker and Gruss, 1993b). Some *Pax* genes encode proteins that contain a complete paired-type homeodomain [the third helix of the paired-type homeodomain has a serine at position nine that confers sequence-specific DNA recognition (Treisman et al., 1989)], but all *Pax* genes encode a DNA binding motif termed the paired domain (Walther et al., 1991). The paired domain encodes 128 amino acids comprising three helixes (Bopp et al., 1986). Several developmental mutations in the mouse have been associated with mutations in *Pax* genes (Hastie,

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1991). A spontaneous mutation located in the paired domain of the *Pax-1* gene has been associated with the mouse developmental mutant *undulated*, which exhibits malformation of the vertebral column. This point mutation leads to a modification in the DNA binding affinity of the mutated protein (Chalepakis et al., 1991) and results in a nontransformant variant of *Pax-1* (Maulbecker and Gruss, 1993b).

We have previously reported the isolation and characterization of a cDNA clone named *Pax-QNR*, found to be the quail homologue of the *Pax-6* gene (Martin et al., 1992; Dozier et al., 1993). A mutation in the *Pax-6* gene has been associated with the mouse *small eye* mutation *Sey* (Hill et al., 1991). The same gene is mutated in the human disease equivalent, the *aniridia* (AN) mutation (Ton et al., 1991), confirming a role for this gene in eye development. The main product of the *Pax-QNR/Pax-6* gene is a 46 kDa protein (Carrière et al., 1993) that specifically binds to the e5 sequence present upstream of the *Drosophila even-skipped*. When expressed in bacteria, both paired and homeodomain peptides recognize e5 (Dozier et al., 1993). The core sequence of most of the paired domain binding sites contains the TTCC (GGAA) sequence (Treisman et al., 1991; Chalepakis et al., 1991; Adams et al., 1992; Fickenscher et al., 1993). We have previously shown that Pax-QNR/Pax-6 is a transcription factor able to transactivate its own promoter (Plaza et al., 1993).

Because members of the Ets family of transcription factors recognize a DNA sequence with a core motif very similar to that of Pax-QNR/Pax-6, we sought to determine whether there exists any interplay between Pax-QNR and members of the Ets family in transcriptional regulation. The GGAA purine core is essential for the specific binding of certain Ets-related proteins to their target sequences (see MacLeod et al., 1992 for review) and we focused our study on two such Ets-gene family members, *Ets-1* and *Erg*. *C-ets-1* ($p68^{c\text{-}ets\text{-}1}$) was of particular interest because this gene is the progenitor of *v-ets*, which is expressed as a fusion protein with *v-myb* by the avian leukemia virus E26 (Leprince et al., 1983, 1988).

In this article we show that $p46^{Pax\text{-}QNR}$ recognizes several well-defined Ets binding sites, and depending on flanking DNA sequence, may transactivate a reporter gene through this sequence. Using a sequence derived from the Polyomavirus enhancer (PyB), we observed that Pax-QNR is not able to transactivate through this sequence, but is able to inhibit Ets-1- or Erg-mediated transactivation. These results suggest possible cross-talk between Ets and Pax family members in gene regulation.

MATERIALS AND METHODS

Cell Culture and Transfection

Quail embryo cells (QEC) prepared from E11 embryos were seeded at 5×10^5 cells per 60-mm dish in DMEM 10% FCS, 24 h prior to transfection. Transfections were performed by the calcium phosphate method. Cells were cotransfected with 1 μg of CAT constructs and 1 or 5 μg of expression vector. The total amount of transfected DNA was kept constant by the addition of vector control. After 4 h, the cells were incubated in fresh medium for an additional 44 h. Cell extracts were prepared and both protein concentration and CAT activity were measured. Each transfection experiment was performed at least twice using two different plasmid preparations to overcome the variability inherent in transfections.

Plasmid Construction

In general, the procedures suggested by Sambrook et al. (1989) were followed. For the MSVtk-CAT plasmid reporter, the double-stranded synthetic oligonucleotide 5′GATCTTCGGAGAGCGGAAGCGCGCGGA3′ was concatemerized in three copies upstream of the minimal tk promoter in the pBL4 vector. To ensure proper orientation, the double-stranded oligonucleotide was multimerized with T4 ligase and digested with BamH1 and BglII restriction endonucleases. The digested DNA was cloned into the BamH1–BglII sites of the intermediate Pic20H vector. The plasmid DNA was then digested by HindIII, and the recovered fragment inserted in the pBL4 vector. For the PyBtkCAT plasmid reporter, the following double-stranded synthetic oligonucleotide was utilized: 5′GATCCTCGACTGTGCTCAGTTAGTCACTTCCTCGA3′. For Gal4tkCAT, the following double-stranded synthetic oligonucleotide was utilized: 5′GATCCGGAAGACTCTCCTCCGA3′. All constructs were subjected to nucleotide DNA sequencing (Pharmacia) to confirm a correct cloning. Erg and Ets-1 expression vectors were described in Duterque-Coquillaud et al. (1993). Pax-QNR/Pax-6 p46 as well as p32 kDa expression vectors were described in Plaza et al. (1993).

Gel Mobility Shift Assays

The DNA probes used were [^{32}P]dCTP labelled with the Klenow fragment of DNA polymerase I (Boehringer). All buffers contained a cocktail of proteinase and phosphatase inhibitors (0.5 mM PMSF, 1 μg/ μl each leupeptin, antipain, pepstatin, 2 mM benzamidine, 10 mM β-glycerophosphate, 2 mM levamisole, 10 μM ortho-vanadate). Binding reac-

tions were performed for 10 min on ice, using 1 ng radiolabelled DNA probe and 1 μg of reticulocyte lysates in 14 μl 10% glycerol, 10 mM HEPES (pH 7.9), 30 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM DTT, 1 mM Na_2HPO_4 (pH 7.2), 1 μg single-stranded salmon sperm DNA, 1.5 μg poly dI-dC. For competition experiments, a 100-fold molar excess of unlabelled competitor oligonucleotides was added simultaneously with the probe. Samples were loaded on a 6% native polyacrylamide gel, run for 2 h at 180 V, and examined by autoradiography after exposure of the dried gel to a Kodak X-AR film at −70°C with an intensifying screen.

In Vitro Transcription and Translation

To synthesize the 46 kDa Pax-QNR, the 68 kDa Ets-1, or 55 kDa Erg proteins in vitro, the relevant pSG5 vectors were transcribed and translated in a rabbit reticulocyte lysate in the presence of T7 polymerase according to the manufacturer's specifications (Promega).

RESULTS

Sequence comparison of the various ets binding sequences described showed that the Moloney Sarcoma Virus Long Terminal Repeat (MSV-LTR) binding site for Ets-1/Ets-2 (Macleod et al., 1992; Gunther et al., 1990) displayed the strongest homology to the paired domain binding site of the e5 sequence (Fig. 1A), and might thus be a target for Pax-QNR/Pax-6 binding. Therefore, we tested the possibility that ^{32}P-labelled MSV-LTR DNA could be specifically recognized by Pax-QNR/Pax-6 expressed in reticulocyte lysate by electrophoretic mobility shift assay (EMSA). Very little endogenous binding activity to the MSV-LTR sequence was found in reticulocyte lysate in which antisense *Pax-QNR* RNA had been translated (Fig. 1B, lane 3), whereas expression of Pax-QNR protein resulted in a shift of the MSV-LTR probe (lane 4). The observed complex was bound specifically to the MSV-LTR sequence because it mostly disappeared in the presence of a 100-fold excess of unlabelled MSV-LTR oligonucleotide competitor (lane 18). As previously described (Duterque-Coquillaud et al., 1993), $p68^{c\text{-}ets\text{-}1}$ or $p55^{erg}$ will retard the MSV-LTR Ets recognition site when tested in a gel shift assay (lanes 1 and 2). The difference in electrophoretic mobility observed for the complexes observed with either Ets-1 or Erg is probably due to the difference in the molecular weight of the proteins. As expected, the $p68^{c\text{-}ets\text{-}1}$ MSV-LTR DNA binding complex was competed by a 100-fold excess of unlabelled MSV-LTR oligonucleotide (lane 15). In contrast, a 100-fold excess of an oligonucleotide containing the AP-1 consensus recognition sequence failed to compete with the MSV-LTR sequence for Pax-QNR or $p68^{c\text{-}ets\text{-}1}$ binding (data not shown). To ascertain the DNA binding ability of the paired domain, we tested in EMSA the DNA binding properties of gel-purified paired domain peptide expressed in bacteria (Dozier et al., 1993). Results show that the paired domain peptide binds to MSV-LTR (lane 5) at least as efficiently as to the e5 sequence (data not shown). Thus, the paired domain alone is able to bind the MSV-LTR oligonucleotide.

Surprisingly, a 100-fold excess of an oligonucleotide containing the e5 recognition sequence competed only partly for $p46^{Pax\text{-}QNR/Pax\text{-}6}$ binding to the MSV-LTR DNA oligonucleotide (lane 19), suggesting that the binding affinity was different for the two oligonucleotides. Indeed, it is well known that the nucleotides surrounding the TTCC or GGAA are important for sequence recognition and binding affinity of Pax (Fickenscher et al., 1993; Adams et al., 1992; Chalepakis et al., 1991; Goulding et al., 1991; Chalepakis et al., 1994) or Ets products (see MacLeod et al., 1992 for review). No binding of the $p68^{c\text{-}ets\text{-}1}$ or $p55^{erg}$ to the e5 sequence could be demonstrated (data not shown), and the $p46^{Pax\text{-}QNR/Pax\text{-}6}$ did not recognize all $p68^{c\text{-}ets\text{-}1}$ binding sites. Among the different Ets-1 binding sites listed in Fig. 1A, $p46^{Pax\text{-}QNR/Pax\text{-}6}$ was able to recognize the PEA3max (lane 9), the PyB sequence (lane 7), and the Py sequence (lane 11), but not the sequence EBS (data not shown). However, Fig. 1C showed that EBS was able to efficiently compete for e5 binding (lanes 2 and 3). This result is reminiscent to the binding efficiency obtained with e5P oligonucleotide (Dozier et al., 1993). This e5 oligonucleotide mutated in the homeodomain binding site was able to efficiently compete for e5 binding, but was unable to form stable complexes with $p46^{Pax\text{-}QNR}$ in EMSA. That the (GGAA)TTCC sequence is important for $p46^{Pax\text{-}QNR}$ binding is demonstrated in Fig. 1D. We tested the binding affinity of Ets binding sites mutated in the GGAA sequence. For example, e5, EBS, MSV, or PEA3max compete efficiently with e5 for $p46^{Pax\text{-}QNR}$ binding; in contrast, PEA3m* or PEA3* (both mutated in the GGAA, see Fig. 1A) compete less efficiently for e5 binding (Fig. 1C,D). Similar results were found with the mutated Ets binding site PyB. As shown in Fig. 1D (lane 9), M1 mutation inserted in the GGAA sequence compete for PyB binding less efficiently than mutations inserted downstream from this site (oligonucleotides M5 and M7). Interestingly, M1 is not recognized by $p68^{c\text{-}ets\text{-}1}$ in EMSA (Wasylyk et al., 1990). In addition, methylation interference analysis on Pax-1

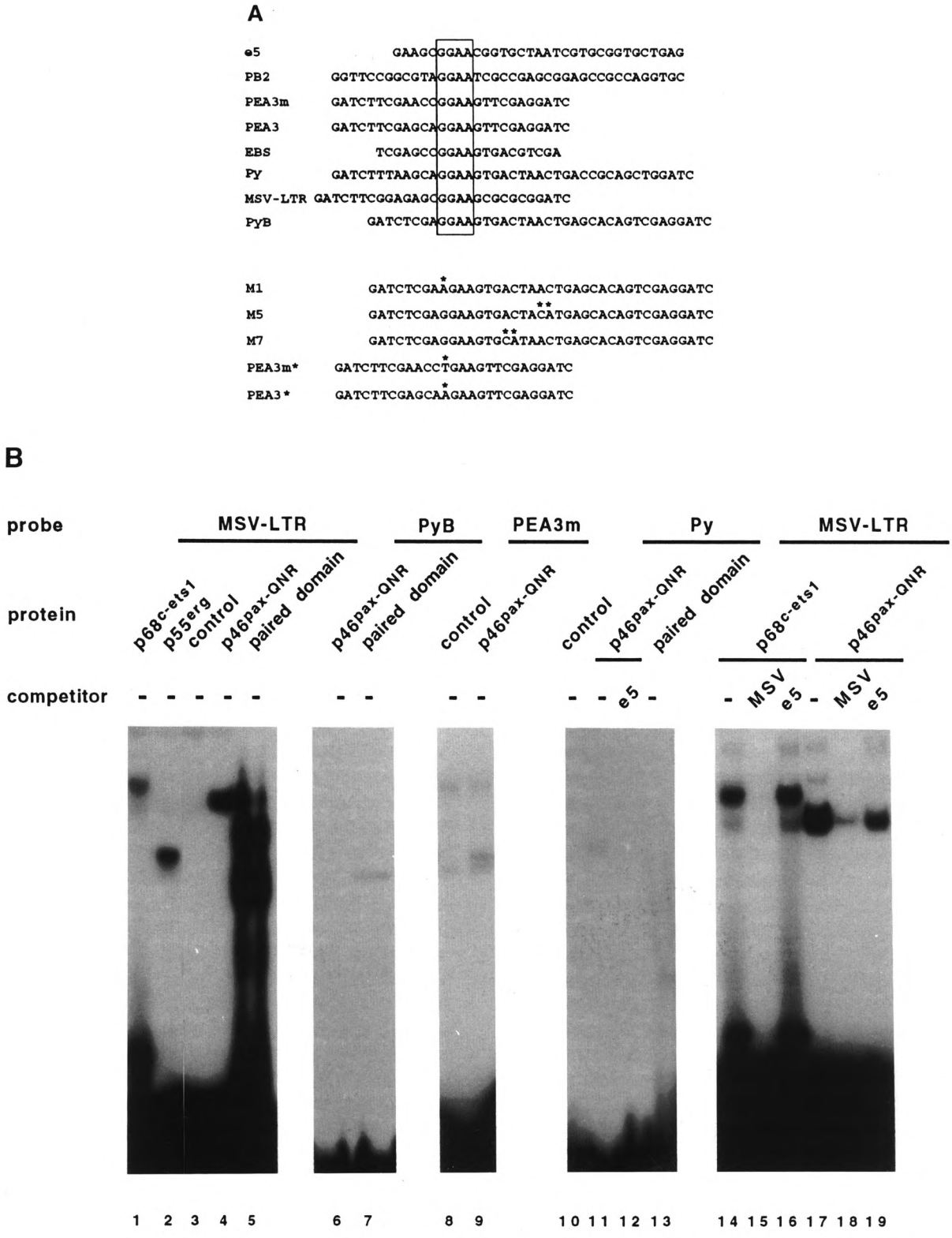

FIG. 1. (A) Comparison of Ets binding (described in MacLeod et al., 1992) and Pax-QNR binding sequences (e5, Treisman, et al., 1991; PB2, Plaza et al., 1993; M1, M5, M7 are mutated version of PyB, Wasylyk et al., 1990). * Shows the mutated nucleotide (B) DNA binding activity of Ets-1, Erg, and Pax-QNR products by electrophoretic mobility-shift assay using different Ets consensus binding sites. The MC29-QNR2 cDNA encoding $p46^{Pax\text{-}QNR}$, cDNA $p55^{erg}$, and cDNA $p68^{c\text{-}ets\text{-}1}$ were transcribed in vitro in a sens and antisens orientation (indicated as control in the top of the figure) and translated in a rabbit reticulocyte lysate. Reticulocyte lysate and ^{32}P-labelled double-stranded oligonucleotide (indicated in the top of the figure) were used for each EMSA reaction. Cold competitor DNA was used in a 100-fold molar excess. The peptides encoded by the paired domain of the Pax-QNR protein were purified after electrophoresis of the bacterial proteins on a 15% NaDodSO4/polyacrylamide gel.

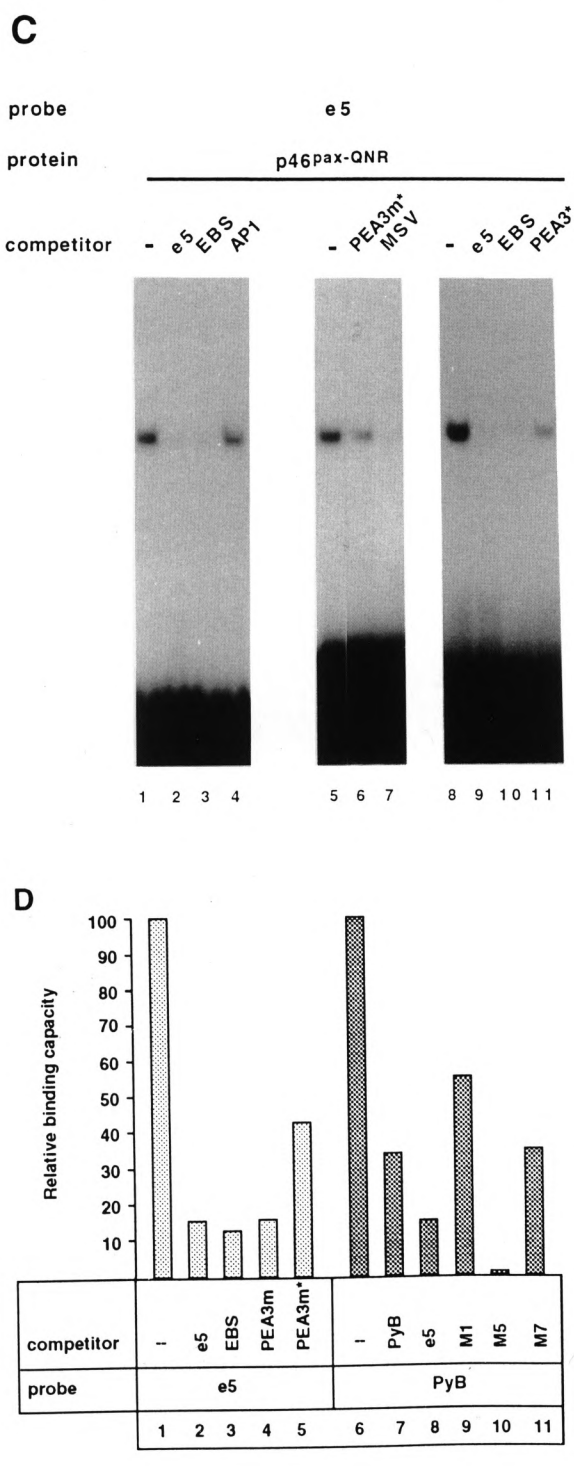

FIG. 1. (C) DNA binding efficiency of Pax-QNR products by EMSA using e5 oligonucleotide and wild-type or mutated Ets binding sites as cold competitor. The MC29-QNR2 $p46^{Pax\text{-}QNR}$ reticulocyte lysate and ^{32}P-labelled double-stranded e5 oligonucleotide was used for EMSA reaction. Cold competitor DNA was used in a 100-fold molar excess. (D) Results of densitometric scanning of EMSA performed as described above with $p46^{Pax\text{-}QNR}$ on ^{32}P-labelled double-stranded oligonucleotides (e5 or PyB) and cold competitor DNA. Signal obtained for each probe without competitor was arbitrarily set at 100. The oligonucleotides used are depicted in (A).

(Chalepakis et al., 1991) or Pax-3 (Chalepakis et al., 1994) binding sites indicate that the TTCC sequence is contacted by the protein, and that the sequence recognized is 20 nucleotides long. Because TTCC is also contacted by Ets proteins (Gunther et al., 1990; Xin et al., 1992), these results suggest that this sequence is the core binding site for both Ets and Pax family members.

When increasing quantities of $p68^{c\text{-}ets\text{-}1}$ were added to a constant amount of $p46^{Pax\text{-}QNR/Pax\text{-}6}$ protein in a binding reaction mixture containing the ^{32}P-labelled MSV-LTR DNA, no ternary complex was obtained (Fig. 2). The same result was obtained with one-tenth of the ^{32}P-labelled MSV-LTR DNA (data not shown). We conclude that at least in vitro the two proteins cannot bind simultaneously to the same DNA molecule.

To study a possible interference between the two proteins in transactivation, we performed transient cotransfection assays in quail embryo cells (QEC) to compare the transcriptional activation of target promoters by $p68^{c\text{-}ets\text{-}1}$ and by Pax-QNR separately, or together. For this purpose we used reporter vectors containing three copies of the MSV-LTR sequences or PyB sequences (Wasylyk et al.,1990) cloned immediately upstream from the minimal tk promoter (MSVtkCAT and PyBtkCAT, respectively). As shown in Fig. 3, cotransfection of the MSVtkCAT with a vector expressing $p46^{Pax\text{-}QNR/Pax\text{-}6}$ (pJ3Ω103) resulted in an increase in CAT activity relative to the vector control (pJ3Ω) (compare lanes 1 and 2). A similarly low increase in CAT activity was obtained with the $p68^{c\text{-}ets\text{-}1}$-encoding expression vector (compare lanes 3 and 4). The $p68^{c\text{-}ets\text{-}1}$-mediated transactivation was not affected when cotransfection was performed with $p46^{Pax\text{-}QNR}$ and $p68^{c\text{-}ets\text{-}1}$ expression vectors (compare lanes 5 and 6), suggesting no synergy or competition between the two proteins on the MSV-LTR sequences. These results suggest that Pax-QNR/Pax-6 can transactivate through an Ets binding site.

We performed similar experiments using the PyBtkCAT construct. However, this vector was not transactivated by the $p46^{Pax\text{-}QNR}$ (Fig. 4A, lanes 7, 8). The basal tk promoter expression was not modified, demonstrating no squelching for basal transcription factors. Thus, although $p46^{Pax\text{-}QNR}$ is able to recognize the Py and PyB sequences in EMSA experiments (Fig. 1B, lane 7 and 11), it is not able to transactivate through the PyB element. In contrast, $p68^{c\text{-}ets\text{-}1}$ (Fig. 4A, lane 6) or $p55^{erg}$ (Fig. 4C, lane 4) were both able to transactivate this reporter construct. This effect is mediated by the 3xPyB sequences because the tkCAT construct alone was not transactivated by expression of $p68^{c\text{-}ets\text{-}1}$ or

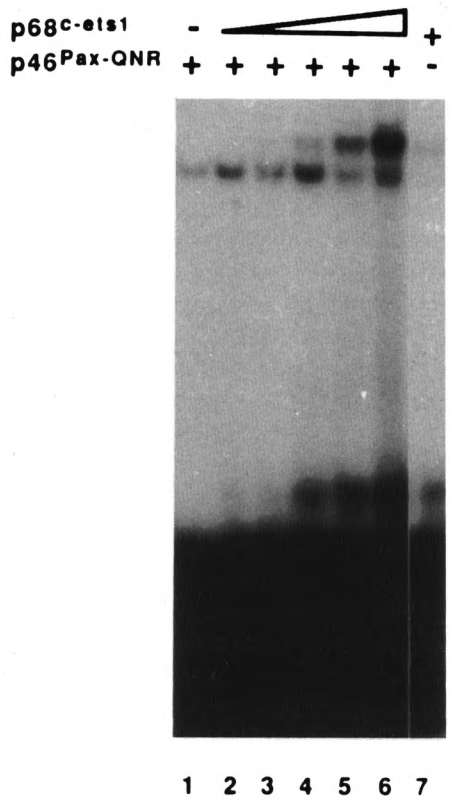

FIG. 2. Pax-QNR and Ets-1 bind to the MSV-LTR oligonucleotide sequence in a mutually exclusive fashion. Increasing amounts (from 0.1 to 2 μl of reticulocyte lysate) of in vitro-translated $p68^{c\text{-}ets\text{-}1}$ were incubated with a fixed amount (0.5 μl, lanes 1 to 6) of in vitro-translated $p46^{Pax\text{-}QNR}$, in a binding assay with MSV-LTR oligonucleotide. Lane 2, 0.1 μl of Ets-1 reticulocyte lysate; lane 3, 0.2 μl; lane 4, 0.5 μl; lane 5, 1 μl; lane 6, 2 μl; lane 7, 0.5 μl of $p68^{c\text{-}ets\text{-}1}$ reticulocyte incubated alone.

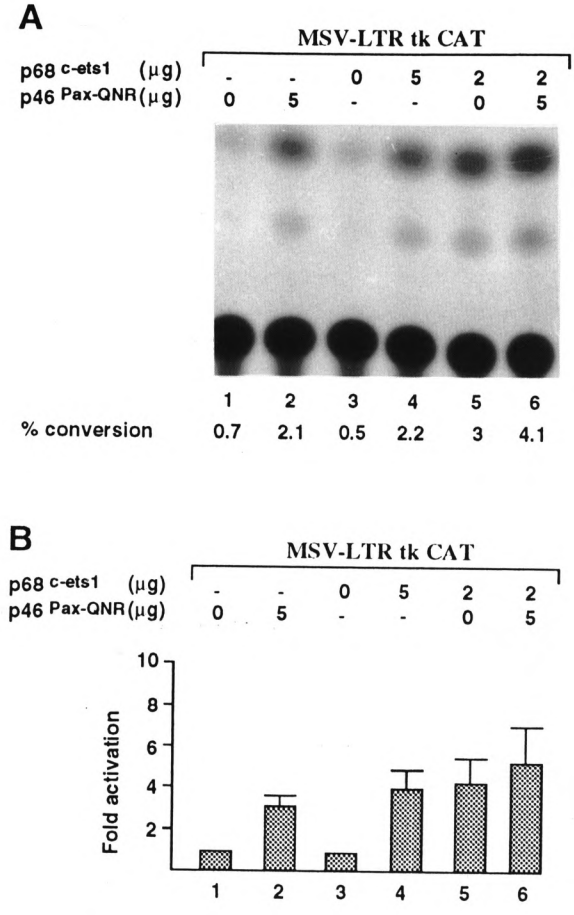

FIG. 3. Comparison of the Ets-1- and Pax-QNR-induced transactivation on 3xMSV-LTRktCAT reporter construct. (A) Quail embryo cells (QEC) were transfected with 1 μg of CAT construct and, when indicated, 5 μg of expression vector. Lane 1, 5 μg of pJ3ΩDNA, expression vector without insert; lane 3, 5 μg of empty pSG5 expression vector; lane 2, pJ3Ω103 containing the MC29-QNR2 cDNA insert, and lane 4, pSG5ets-1 vector expressing the $p68^{c\text{-}ets\text{-}1}$. Lane 5, 2 μg of pSG5ets-1 and 5 μg of pJ3ΩDNA; lane 6, 2 μg of pSG5ets-1 and 5 μg of pJ3Ω103 DNA. Numbers below lanes indicate the percentage of conversion of acetylated derivatives. (B) Because it was not possible to normalize the transfection with a cotransfected plasmid (due to plasmid backbone response), we repeated four independent transfections with two distinct DNA preparations. The activity of the MSV-LTRtkCAT reporter in the absence of exogenous transactivator was arbitrarily designated as 1. Thin lines indicate standard deviations.

$p46^{Pax\text{-}QNR}$ (Fig. 4A; lanes 2 and 4). We therefore tested whether Pax-QNR could interfere in the promoter transactivation by the Ets proteins through the PyB sequence. As shown in Fig. 4A (lanes 9 to 12), coexpression of $p46^{Pax\text{-}QNR}$ with $p68^{c\text{-}ets\text{-}1}$ strongly reduced $p68^{c\text{-}ets\text{-}1}$-induced transactivation (compare lane 11 with lanes 10 and 12, Fig. 4A and B). We found the same effect on $p55^{erg}$ transactivation (Fig. 4C, lanes 5 and 6) expression of $p46^{Pax\text{-}QNR}$ strongly reduced $p55^{erg}$-induced transactivation (compare lane 5 and lane 6). To confirm that this transactivation interference is restricted to Ets family members, we analyzed the effect of $p46^{Pax\text{-}QNR}$ on VP16-induced transactivation. The transactivating protein contains the carboxyl-terminal transcriptional activation domain of the VP16 protein of HSV (Triezenberg et al., 1988) and the DNA binding domain of the yeast transcriptional activator GAL4 (Kakidani and Ptashne, 1988). As shown in Fig. 4A, GAL4-VP16 was able to transactivate a reporter plasmid bearing four GAL4-binding sites upstream of the tk minimal promoter (lanes 14 and 17), and this transactivation was not significantly affected by the cotransfection of Pax-QNR-encoding expression vector (compare lanes 17 and 18). Therefore, the interference between Pax-QNR and the two Ets family members tested in PyBtkCAT transactivation is not the result of a nonspecific repression of activated transcription, but is specific to Ets-induced transactivation. To study whether Pax-QNR binding activity is required for interference with transactivation by Ets, we used a

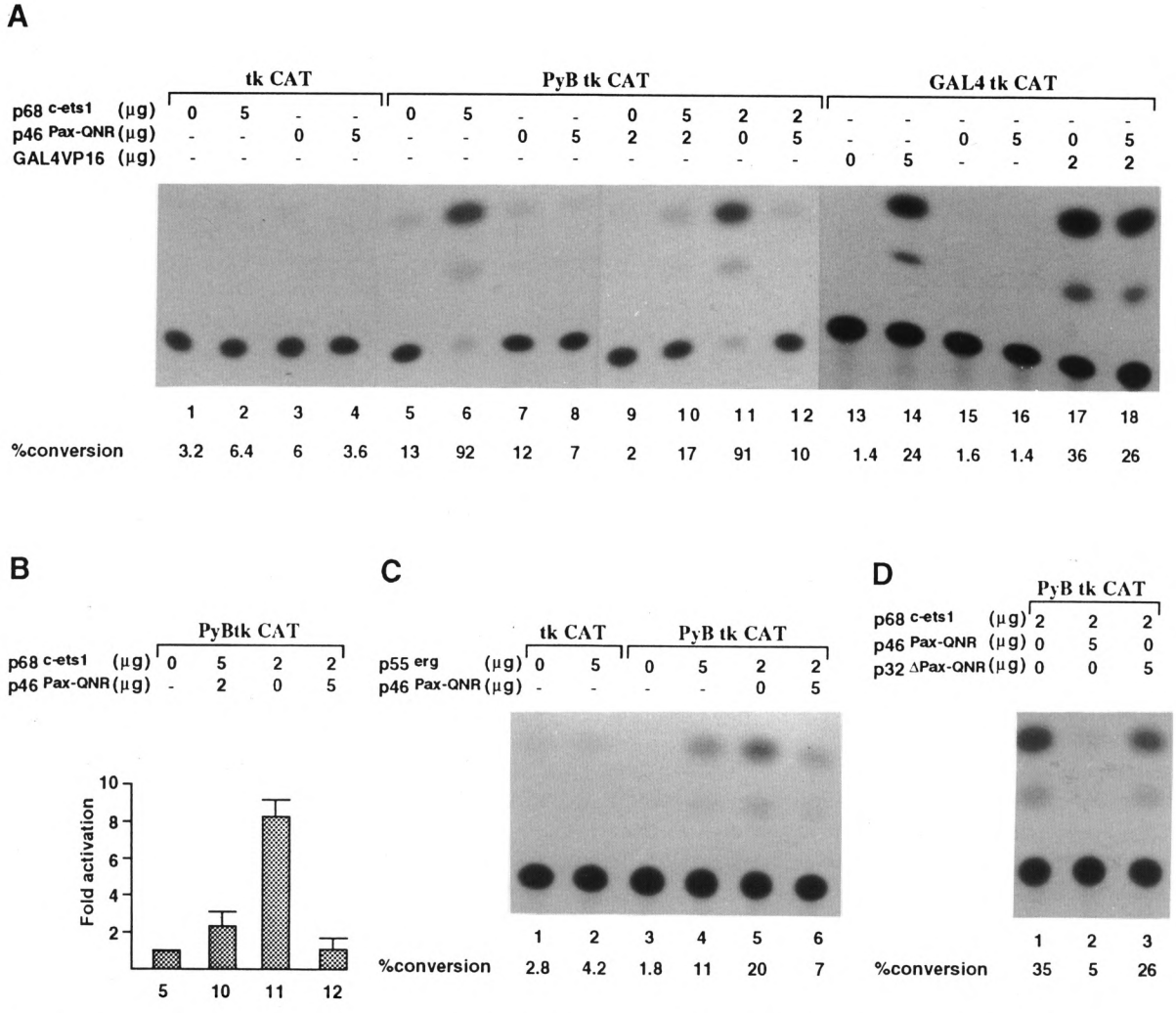

FIG. 4. Interference of the Ets-induced transactivation by the Pax-QNR protein on 3xPyBtkCAT reporter construct. (A) QEC were transfected with 1 µg of CAT constructs and, when indicated, 5 µg of PJ3Ω103 expressing the $p46^{Pax\text{-}QNR}$, pSG5ets-1 expressing $p68^{c\text{-}ets\text{-}1}$, and pSG424-GAL4/VP16 encoding the GAL4 DNA binding domain (Sadowsski and Patshne, 1989) fused to the VP16 transactivation domain (vector kindly provided by A. Leutz). The total amount of DNA in the transfection was kept constant by addition of the vector-control, pJ3Ω or pSG5. The CAT activities were performed using equal amounts of proteins. Control reporter plasmids include the tkCAT without the 3xPyB sequence (lanes 1 to 4) and the GAL4tkCAT (4xGAL4 DNA binding sequence) lanes 13–18. Numbers below lanes indicate the percentage of conversion of acetylated derivatives. (B) Average values and standard deviations on CAT activities using DNA indicated on the top of the figure; a representative experiment is shown in (A). We repeated four independent transfections with two distinct DNA preparations. The activity of the PyBtkCAT reporter in the absence of exogenous transactivator was arbitrarily set at 1. Thin lines indicate standard deviations. (C) QEC were transfected with 1 µg of CAT constructs and, when indicated, 2 or 5 µg of expression plasmid, pJ3Ω103 or pSG5erg expressing $p55^{erg}$. Numbers below lanes indicate the percentage of conversion of acetylated derivatives. (D) QEC were transfected with 1 µg of CAT constructs and, when indicated, 2 µg of expression plasmid pSG5ets-1, and 5 µg of pJ3Ω103 or pSG5ΔNhe expressing the p32 Pax-QNR paired-less protein (Carrière et al., 1993). Quantification of the CAT assays with an instant imager (Packard) indicates an average of 10-fold increase in CAT activity with Ets-encoding vectors. Numbers below the lanes indicate the percentage of conversion of acetylated derivatives.

form of Pax-QNR protein lacking the paired domain in cotransfection assays. This p32 paired-less protein is devoid of DNA binding properties (Carrière et al., 1993) and unable to transactivate Pax-QNR targets (Plaza et al., 1993). The Pax-QNR p46 and p32 are produced in the same amount in the transfected cells and both proteins can be detected into the nucleus (Plaza et al., 1993; Carrière et al., 1993; and data not shown). As shown in Fig. 4D, the paired-less proteins have no effect on the $p68^{c\text{-}ets\text{-}1}$-induced by PyBtkCAT transactivation (compare lanes 1 and 3), in contrast to the full-length Pax-QNR protein (lane 2). Thus, efficient DNA binding is required to interfere with Ets-induced transactivation, and this interference is probably mediated by competition for DNA binding sites.

DISCUSSION

Molecular control of differentiation processes such as lineage commitment and cellular maturation is likely to hinge upon lineage-restricted transcription factors that induce or repress batteries of subordinate genes. Like Pax family members, Ets proteins are tissue specific and developmentally regulated transcription factors (see MacLeod et al., 1992). In this article we have found that Ets family members and Pax-6 are able to bind in vitro to the same DNA sequences, and depending on the sequence environment, to transactivate identical promoter constructs or to interfere at the transcriptional level. The relaxed DNA binding specificity of Ets-1 (Bosselut et al., 1993) suggests that determinants other than the nucleotide sequence are likely to determine whether the Ets-1 DNA binding site will respond functionally. Mechanisms probably exist that modulate the activity of these sites. Binding of Ets family members to select promoter/enhancer sequences has been shown to occur in conjunction with other nuclear factors, possibly through protein–protein interactions (Dalton and Treisman, 1992; Pogubala et al., 1992; Thompson et al., 1991). Activation of the Polyoma virus enhancer by Ets-1 occurs in synergy with AP1 (Jun/Fos) (Wasylyk et al., 1990), and activation of the HTLV-1 LTR by Ets-1 occurs in synergy with Sp1 (Gégonne et al., 1993). All these data suggest that Ets family members function as components of larger transcription complexes to regulate the activity of target genes.

The observed interaction between $p46^{Pax\text{-}QNR}$ and $p68^{c\text{-}ets\text{-}1}$ or $p55^{erg}$ at the transactivation level suggests a competition between these proteins for the binding to the PyB sequence. That $p46^{Pax\text{-}QNR}$ abrogates $p68^{c\text{-}ets\text{-}1}$ or $p55^{erg}$ expression is unlikely. We found that the Ets binding site present in HTLV1 LTR sequence is transactivated by $p68^{c\text{-}ets\text{-}1}$ but not by $p46^{Pax\text{-}QNR}$, and no interference between these proteins could be observed. The $p68^{c\text{-}ets\text{-}1}$ protein is still able to transactivate the HTLV-1 LTR even in the presence of Pax-QNR encoding vector (data not shown). Thus, binding of $p46^{Pax\text{-}QNR}$ to PyB sequences may prevent binding of transactivating Ets proteins by a simple mechanism such as physical occlusion. We demonstrated that $p46^{Pax\text{-}QNR}$ is able to transactivate through specific Ets-recognized DNA sequences (MSV-LTR), even if the transactivation is weak, but not weaker than for $p68^{c\text{-}ets\text{-}1}$. Therefore, the lack of response of PyBtkCAT to $p46^{Pax\text{-}QNR}$ is either due to the need of additional factors in the initiation complex or to the need of a particular spacing of the DNA binding sequence with respect to the TATA box for a transcriptional response. Competition for binding to PyB sequence between $p46^{Pax\text{-}QNR}$ and $p68^{c\text{-}ets\text{-}1}$ or $p55^{erg}$ is further suggested by the lack of interference between the DNA binding-deficient paired-less $p32^{Pax\text{-}QNR}$ protein and $p68^{c\text{-}ets\text{-}1}$. We have shown previously that this particular isoform was unable to bind Pax-QNR target DNA sequences or to transactivate the *Pax-QNR* promoter (Carrière et al., 1993; Plaza et al., 1993). Protein–protein interactions through dimerisation domains such as the bZIP motif are a general mechanism by which DNA binding activity and biological functions of heterodimeric proteins are modified (Glass et al., 1989; Bengal et al., 1992; Stein et al., 1993; Ayer and Eisenman, 1993). However, we have found no evidence for any physical interaction between $p46^{Pax\text{-}QNR}$ and $p68^{c\text{-}ets\text{-}1}$ in vitro (data not shown).

Dominant negative repressors with transforming properties have evolved from a class of genes encoding ligand-regulated transcription factors. As a consequence of its imprecise copy of the cellular gene (*c-erbA*), the oncogenic version of thyroid hormone receptor (v-erbA) can bind to DNA but, unlike c-erbA, cannot activate transcription in response to hormone; instead, the v-erbA protein blocks transcriptional activation mediated by the c-erbA polypeptide (Damm et al., 1989) and can also repress retinoic acid action (Sharif and Privalsky, 1991). The cAMP-responsive element modulator (CREM) encodes both antagonists and an activator of the cAMP transcriptional response by alternative splicing, and CREM antagonists are able to block the transcriptional activation elicited by the c-jun and other Jun family members (Masquilier and Sassone-Corsi, 1992). The downregulation is likely to be obtained by occupation of the DNA binding sites for c-Jun because CREM proteins do not heterodimerize with c-Jun (Masquilier and Sassone-Corsi, 1992). However, in contrast to the proteins mentioned above, Ets and Pax DNA binding domains are clearly distinct. Computer structure predictions suggest the presence of three α-helices in the paired domain (Bopp et al., 1986). The Ets DNA binding domain is also predicted to contain three α-helices (Bosselut et al., 1993) and Hydrophobic Cluster Analysis suggests that the Ets domain is likely to contain two helix–loop–helix motifs (Laget et al., 1993). In addition, Pax (Czerny et al., 1993) and Ets (Fisher et al., 1992; Nye et al., 1992) transcription factors most probably bind to DNA as monomers. Ets-1/DNA backbone interactions span a 20-nucleotide region and are localized on one face of the helix: contacts map to the major groove, in the center of the site (Nye et al., 1992). The Pax-5 paired domain binds to two half-sites of the target sequence in adjacent major grooves on the same side of the

DNA helix (Czerny et al., 1993). Pax-1 recognizes a 24 bp recognition sequence as monomer, which includes minor groove, DNA backbone, and major groove contacts (Chalepakis et al., 1991). Therefore, even if Pax and Ets DNA binding domains are distinct, they exhibit some common features that may explain in part the observed interaction of Ets-1 and Pax-QNR with the PyB binding site. Further structural analyses are required to resolve the molecular details of Ets/Pax DNA interactions.

This interaction implies common gene regulation. Unfortunately, target genes for Pax-QNR/Pax-6 products are not yet defined. *Ets* genes are proto-oncogenes and Pax members have been recently found to transform immortalized murine fibroblasts (Maulbecker and Gruss, 1993b). Therefore, genes involved in the control of cell growth could be potential targets for *Ets* and *pax* gene families. Among such genes, *c-fos*, involved in many aspects of cell proliferation (see Rivera and Greenberg 1990 for review) appears a good candidate. Among the numerous regulatory sequences surrounding the *c-fos* promoter is the serum response element (SRE), which binds several proteins and plays a key role in the serum response of the gene (Rivera and Greenberg, 1990; Treisman, 1990). The SRE binds SRF (Serum Response Factor, Treisman, 1990), an Ets family member $p62^{Elk-1}$ (Hipskind et al., 1991), and also a paired-type homeobox protein PHOX (Grueneberg et al., 1992). These proteins interact with each other and DNA sequences (Shaw, 1992; Grueneberg et al., 1992). We have found that the SRE sequence is recognized in EMSA by the $p46^{Pax-QNR}$ protein (data not shown). Thus, the potential interaction between Pax-6 and Elk-1 should be investigated to examine their role in *c-fos* regulation.

Another area to investigate a possible Ets/Pax interaction is the induction of angiogenesis. As pointed out by Maulbecker and Gruss (1993b), Pax-6-induced tumors are highly vascularized and, because Ets-1 is expressed in endothelial cells and modulated in response of angiogenic factors (Véronique Fafeur, personal communication), it will be interesting to investigate Ets/Pax interactions in this cellular system.

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