Dissection of the Transactivation Function of the Transcription Factor Encoded by the Eye Developmental Gene PAX6

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PAX6 is a transcription activator that regulates eye development in animals ranging from Drosophila to human. The C-terminal region of PAX6 is proline-serine/threonine-rich (PST) and functions as a potent transactivation domain when attached to a heterologous DNA-binding domain of the yeast transcription factor, GAL4. The PST region comprises 152 amino acids encoded by four exons. The transactivation function of the PST region has not been defined and characterized in detail by in vitro mutagenesis. We dissected the PST domain in two independent systems, a heterologous system using a GAL4 DNA-binding site and the native system of PAX6. Our data consistently showed that in both systems all four constituent exons of the PST domain are responsible for the transactivation function. The four exon fragments act synergistically to stimulate transcription, although none of them can function individually as an independent transactivation domain. Combinations of two or more exon fragments can reconstitute substantial transactivation activity when fused to the DNA-binding domain of GAL4, but they surprisingly do not produce much activity in the context of native PAX6, although the mutant PAX6 proteins are stable and their DNA-binding function remains unaffected. Our data suggest that these mutants may antagonize the wild-type PAX6 activity by competing for target DNA-binding sites. We conclude that the PAX6 protein contains an unusually large transactivation domain that is evolutionarily conserved to a high degree and that its full transactivation activity relies on the synergistic action of the four exon fragments.

Aniridia is a congenital eye disorder characterized by complete or partial absence of the iris (1–2). The gene responsible for aniridia in humans, PAX6, was isolated by positional cloning (3) and documented by mutations found in both familial and sporadic aniridia (4–10). The PAX6 gene encodes a 422-amino acid (aa)1 protein that has paired box and homeobox motifs. The paired box motif, originally identified in the Droso-

1 The abbreviations used are: aa, amino acid(s); GAL4-DDB, GAL4 DNA-binding domain; PD, paired domain; HD, homeodomain; PST, proline-serine/threonine-rich; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; E10–E13, exons 10–13; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus.

2 Summarized on the World Wide Web at http://craigellachie.hgu.mrc.ac.uk/Softdata/PAX6/.

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MATERIALS AND METHODS

Cell Culture—HeLa, a human cervical carcinoma cell line (CCL2, ATCC), was maintained in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. NIH 3T3, a murine fibroblast cell line, was maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum. HEB3, a human lens epithelial cell line (38), was maintained in Eagle’s minimal essential medium supplemented with 20% fetal calf serum.

Plasmid Constructs—GAL4-PAX6 fusion expression vectors were generated by polymerase chain reaction (PCR) cloning. In brief, specific regions of PAX6 were amplified by PCR using the cDNA plasmid clone ph12 (3) as a template. The primers used are listed in Table I. All 5’-end primers contained an XbaI restriction site. All 3’-end primers contained a BamHI restriction site. The PCR products were digested with endonucleases XbaI and BamHI, inserted into the XbaI–BamHI restriction sites of the expression vector PCGGAL (39), and then fused in-frame to the N-terminal GAL4 DNA-binding domain (G4; aa 1–97).

pRe-CMV-PAX6 expression plasmids were constructed by a similar PCR cloning strategy. Specific regions of PAX6 were amplified by PCR using the cDNA clone ph12 (3) as a template. Primers used to amplify specific regions of PAX6 are listed in Table I. All 5’-end primers contained a BamHI restriction site. All 3’-end primers contained a SpeI restriction site and a stop codon. Endonuclease digestion with SpeI and XbaI generated compatible cohesive ends. The HindIII–SpeI-digested PCR products were ligated into the HindIII–XbaI restriction sites in the polylinker region of the expression vector pRe-CMV (Invitrogen).

The PAX6-SacII construct used to make internal deletions by site-directed mutagenesis was created by recombinant PCR (40). The 5’-primer contained a HindIII restriction site and a Kozak consensus sequence. The 3’-primer contained a HindIII restriction site and a Kozak consensus sequence. The 3’-primer contained a stop codon followed by an XbaI restriction site. The two internal mutant primers contained a SacII restriction site designed to fit in-frame between aa 277 and 278 of PAX6. All of the internal deletion constructs were constructed using a common 3’-primer that contained an XbaI restriction site but different 5’-primers that all contained a SacII restriction site. All of the internal deletion constructs were inserted between the SacII and XbaI restriction sites of the PAX6–SacII construct to create various internal deletion mutants.

The PAX6-(4V/G) construct was similarly created by recombinant PCR using primers designed to mutate Val401, Val403, Val405, and Val407 into glycines simultaneously.

Transfections—HeLa and NIH 3T3 cells were plated at a density of 4–6 × 10⁵ cells/60-mm tissue culture dish 24–36 h prior to transfection. Transfections for all cells were carried out with plasmid DNA coated with the polycationic lipid lipofectamine (Life Technologies, Inc.) according to the manufacturer’s instructions. HeLa cells were transfected at 50–70% confluence using 6 μl (12 μg) of lipofectamine/60-mm dish.

| Name of constructs | Primer sequence |
|--------------------|-----------------|
| G4P                | 5’-CGGCTCTAGAATG-CAGAAGCTCACGCGGA-3’ |
| G4PG               | 5’-GCGGGATCCTAGAGCAACAGATTAAGGAATCATCT-3’ |
| G4PGH              | 5’-GCGGGATCCTATTTCTTCTTCTTCCATTT-3’ |
| G4PGHP             | 5’-GCGGGATCCTACTGAAAGAAATAGAACAT-3’ |
| G4GH               | 5’-GCGCTCTAGACTGCAAAGAAATAGAACAT-3’ |
| G4GP               | 5’-GCGGGATCCTACTGAAAGAAATAGAACAT-3’ |
| G4H                | 5’-GCGGGATCCTACTGAAAGAAATAGAACAT-3’ |
| G4Pst              | 5’-GCGCTCTAGAATCAGAGACAGACCCACGCA-3’ |
| G4G                | 5’-GCGGGATCCTACTGAAAGAAATAGAACAT-3’ |
| G4E10              | 5’-GACTAGTCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E11              | 5’-GACTAGTCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E12              | 5’-GACTAGTCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E13              | 5’-GACTAGTCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E10+11           | 5’-GCACTGCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E11+12           | 5’-GCACTGCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E12+13           | 5’-GCACTGCGAGATTCTCTTCCATTTCCACT-3’ |
| G4E10+11+12        | 5’-GCACTGCGAGATTCTCTTCCATTTCCACT-3’ |
NIH 3T3 cells were transfected at 60–80% confluence using 15 μl (30 
mg) of lipofectamine/60-mm dish. HEB3 cells were transfected at 70–
80% confluence using 6 μl (12 
mg) of lipofectamine/60-mm dish. For the
GAL4 fusion experiments, each dish was transfected with 0.8 μg of reporter plasmid 4
GAL4-fos-
CAT, 0.025–0.4 μg of GAL4 effector
plasmid, 0.8 μg of the internal control plasmid pSV2
β-galactosidase (Promega), and pBluescript carrier plasmid (Strategene, La Jolla, CA) to bring the total amount of plasmids to 3.2 μg. For transfections with
the pRc-CMV-PAX6 expression vectors, each dish was transfected with
2 μg of reporter plasmid P6CON-
CAT (41) or CD19–2-(A-ins)-luciferase
(21), 1 μg of pRc-CMV effector plasmid, and 0.4 μg of pSV2 β-galacto-
sidase (Promega) as internal control.

Chloramphenicol Acetyltransferase (CAT) Assay—
Cell extracts were
prepared after 24–72 h of transfection. The cells on each 60-mm culture
dish were rinsed twice with ice cold 1
3 Dulbecco’s phosphate-buffered
saline, detached using a rubber policeman into 160 μl of ice-cold 0.25
mM Tris, pH 7.8, and then transferred into a microcentrifuge tube. The
cells were then lysed by three cycles of freezing in dry ice and ethanol
and thawing at 37 °C. The microcentrifuge tube was then centrifuged in
a microcentrifuge at full speed for 5 min at 4 °C, after which the
supernatant was transferred to a fresh microcentrifuge tube and used
immediately or stored at
270 °C for future use. The cell extracts were
first assayed for
β-galactosidase activity and then normalized by dilu-
tion with 0.25 mM Tris, pH 7.8. CAT assays were performed according
to standard procedures (55), and extracts were diluted as necessary to
keep the assay in the linear range. The percentage of acetylation of
[14C]chloramphenicol was quantitated directly from thin layer chroma-
tography plates using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Luciferase and β-Galactosidase Assays—
Cell extracts were prepared after 24–48 h of transfection. The cells on each 60-mm tissue culture
dish were rinsed twice with 3 ml of 1
3 phosphate-buffered saline and 
lysed at room temperature in 250 μl of lysis solution (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol added fresh
prior to use). The cell lysates were detached from the culture plate using
a rubber policeman or cell scraper, transferred to a microcentrifuge
tube, and centrifuged for 2 min to pellet any debris. Supernatant was
transferred to a fresh microcentrifuge tube. Cell extracts were then
used immediately for luciferase and β-galactosidase assays or frozen at
270 °C for future use.

Luciferase activity was measured at room temperature using a lu-
ciferase assay kit (Tropix, Bedford, MA; catalog number BC100L). All
reagents and cell extracts were warmed to room temperature before use. Briefly, 50 μl of substrate A was aliquoted into 75 × 12-mm luminometer sample tubes (Sarstedt; catalog number 55.476). Then 10 μl of each cell extract and 50 μl of substrate B were added sequentially into the tube, which was then placed immediately into a luminometer for measurement. The luminometer was set to measure the luciferase signal for 10 s with a 2-s delay.

β-Galactosidase activity was detected using a Galacto-Light Plus

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### Table II

| Name of constructs | Primer sequence |
|--------------------|-----------------|
| PAX6(1-422)        | 5’ primer....5’-GCCCAAGCTTCCAGCATGCAGACGT-3’ |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6(1-413)        | 5’ primer.... same as for PAX6(1-422). |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6(1-404)        | 5’ primer.... same as for PAX6(1-422). |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6(1-395)        | 5’ primer.... same as for PAX6(1-422). |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6(1-344)        | 5’ primer.... same as for PAX6(1-422). |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6(1-306)        | 5’ primer.... same as for PAX6(1-422). |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6(1-272)        | 5’ primer.... same as for PAX6(1-422). |
|                    | 3’ primer....5’-GGACTAGTCTTTAGTTATCGGGCAG-3’ |
| PAX6-SacII         | 5’ primer.... same as for PAX6(1-422). |
| SacII primer-sense.| 5’-AGGAATCAGAGAGACCCCGGGGCAACACACACT-3’ |
| SacII primer-antisense.| 5’-AGGTGTTGTTGCGGGGCTGGTATCGTCTACTACAT-3’ |
| PAX6EII+12+13      | 5’ primer....5’-TCCCCGCCGTTCTCTCTACATCTGGTC-3’ |
|                    | 3’ primer.... same as for PAX6-SacII. |
| PAX6EII+13         | 5’ primer....5’-TCCCCGCCGTTCTCTCTACATCTGGTC-3’ |
|                    | 3’ primer.... same as for PAX6-SacII. |
| PAX6EII13          | 5’ primer....5’-TCCCCGCCGTTCTCTCTACATCTGGTC-3’ |
|                    | 3’ primer.... same as for PAX6-SacII. |
| PAX6(4V/G)         | 5’ primer.... same as for PAX6(1-422). |
| 4V/G primer-sense. | 5’-CTCATTTCCCTGGTGCTAGTCAAGTTGCTCCGGAGATGACCT-3’ |
| 4V/G primer-antisense.| 5’-AGGTTCACTTCCGGAACCTTGAACAGCTACAAAGACATGAGATAAAAGTGGAGATCCAGAAGAGC-3’ |
| Flag-PST           | 5’ primer....5’-GCCCAAGCTTCCAGCATGCAGACGTACAAAGACATGAGATAAAAGTGGAGATCCAGAAGAGC-3’ |
|                    | 3’ primer.... same as for PAX6-SacII. |
| HA-PST             | 5’ primer..5’-GCCCAAGCTTCTTGGTAGACCTACAAAGACATGAGATAAAAGTGGAGATCCAGAAGAGC-3’ |
|                    | 3’ primer.. same as for PAX6-SacII. |
chemiluminescent assay kit (Tropix; catalog number BL100G). All reagents and cell extracts were warmed to room temperature before use. Briefly, 200 μl of reaction buffer containing Galacton-Plus substrate was dispensed into 75 × 12-mm luminometer sample tubes. Then, 5 μl of individual cell extracts was added to the tube, which was incubated at room temperature for 1 h. Then, 300 μl of Accelerator reagent was added to each tube, which was immediately placed in a luminometer for measurement. The measurement protocol was the same as for the luciferase assay. Luciferase activities were normalized relative to β-galactosidase activity.

**Nuclear Extract Preparations**—Crude nuclear extracts were prepared from transfected HeLa or NIH 3T3 cells according to the method of Schreiber et al. (42), as modified by Singh and Aggarwal (43). The cells were harvested from each 60-mm culture dish and lysed in 0.2 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 mM leupeptin, 2.0 mM aprotinin, and 0.5 mg/ml benzamidine). The nuclear pellet was resuspended in 25 μl of ice-cold extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.0 mg/ml leupeptin, 2.0 mg/ml aprotinin, and 0.5 mg/ml benzamidine). The protein concentration of each nuclear extract was measured by Bradford assay.

**Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays (EMSAs) were performed in a 20-μl binding reaction incubated at room temperature for 30 min in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 0.1 mg/ml poly(dI-dC), and 16 fmol of probes labeled with 32P). Protein-DNA complexes were resolved on a 4.5% native polyacrylamide gel using a buffer containing 50 mM Tris, 200 mM glycine, pH 8.5, and 1 mM EDTA (43). The gel was analyzed by a PhosphorImage using ImageQuant software (Molecular Dynamics). For supershift assays, the binding reaction mixture was incubated with the monoclonal antibody against PAX6-(16–422) for 30 min at room temperature before loading onto the gel.

**Western Blotting Assay**—The nuclear extracts prepared from transfected HeLa, NIH 3T3, or HEB3 cells were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and then analyzed for the presence of proteins of the expected sizes by an immune serum raised against the GAL4-fos-CAT protein (ECL kit, Pharmacia Amersham Biotech). The bands on the Western blots were quantitated using Personal Densitometer Scan version 1.30 and ImageQuant Version 3.3 software (Molecular Dynamics).

**RESULTS**

**Transactivation Function of PAX6**—The identification and characterization of functional protein domains are critical to understanding the molecular role of PAX6. We (28) and others (21, 29) have demonstrated previously that the PST region of truncated PAX6 can confer a potent transactivation function when fused to the DNA-binding domain of the yeast transcription activator GAL4(1–97) and tested for their ability to transactivate the reporter gene 4 CAT, which contains four GAL4 binding sites inserted into the pRc-CMV expression plasmid and fused to an N-terminal Flag epitope tag. Some of the GAL4 fusion constructs, including the GAL4-DBD and GAL4-fos-CAT, produced very strong activity when expressed by hybridization with polyclonal antibodies raised against the paired domain (PD) of PAX6 (aa 1–127) (44). The protein band was detected by enhanced chemiluminescence using a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (ECL kit, Pharmacia Amersham Biotech). The bands on the Western blots were quantitated using Personal Densitometer Scan version 1.30 and ImageQuant Version 3.3 software (Molecular Dynamics).

**Transactivation Function of PAX6**—The identification and characterization of functional protein domains are critical to understanding the molecular role of PAX6. We (28) and others (21, 29) have demonstrated previously that the PST region of truncated PAX6 can confer a potent transactivation function when fused to the DNA-binding domain of the yeast transcription activator GAL4(1–97). To examine the functional relationship of the PST region with other domains, we fused full-length PAX6 to the DNA-binding domain of GAL4 and tested its ability to activate transcription from a promoter bearing GAL4 binding sites. The reporter plasmid, 4 × GAL4-fos-CAT, contains four GAL4 binding sites inserted upstream of the c-fos TATA box linked to the CAT gene. Cotransfection of the effecter plasmid and the reporter plasmid into HeLa cells revealed that the full-length PAX6 fused with GAL4 did not show transcriptional activation (Fig. 1A), whereas deletion of the PD resulted in an efficient transcriptional activation of the CAT gene. This suggests that the presence of the PD could either interfere with the DNA binding of GAL4 or conformationally interfere with the interaction between the PST domain and the general transcriptional machinery. Further deletion of the linker region (G) caused a reduction in transactivation. This reduction was likely due to the negative effects of the homeodomain (HD), which became stronger after removal of the G region or when the HD was placed in proximity to GAL4. Although the function of the G region is unknown, it is not directly involved in transactivation, since this region had no activity when tested either alone or in association with the PD or HD. Finally, deletion of the entire PD-G-HD region showed that the C-terminal 152-aa PST region produced the highest activation (Fig. 1A). In contrast, removal of the PST region resulted in the complete loss of transactivation in all C-terminal deletion mutants. These results demonstrate that the transactivation function of PAX6 resides in the C-terminal PST region, which can function independently of the other regions of PAX6 when fused with an appropriate DNA-binding domain. They also show that PD and HD have negative effects on the transactivation function of the GAL4-PAX6 fusion protein. Similar negative effects of PD and HD have been observed with the GAL4-Pax3 fusion protein (31).

**Dissection of the PST Region**—To dissect the transactivation domain, we divided the PST region at the exon boundaries into four fragments: E10 (composed of 35 aa), E11 (39 aa), E12 (50 aa), and E13 (28 aa). These four exons were fused individually to GAL4(1–97) and tested for their ability to transactivate the reporter gene 4 × GAL4-fos-CAT in HeLa cells (Fig. 2B). None of the four exon fragments caused significant activation, indicating disruption of the transactivation domain. To verify the protein expression of the transfected GAL4 fusion constructs, we performed Western blot analysis of nuclear extracts prepared from HeLa cells transfected under the same experimental conditions as used for CAT assays. The GAL4 fusion proteins of the expected sizes were detected in the nuclear extracts by an immune serum raised against the GAL4 DNA-binding domain (GAL4-DBD) (data not shown). We next tested combinations of adjacent exons: E10 and E11 of 74 amino acids, E11 and E12 of 89 amino acids, and E12 and E13 of 78 amino acids. All three combinations resulted in synergistic activation at similar levels, albeit with more activity shifted to the C-terminal exons. Further combination of three exons, E10, E11, and E12, produced higher activity than combination of two exons. Protein expression of transfected constructs was verified by Western blot using the polyclonal antibody against the GAL4-DBD (data not shown). Some of the GAL4 fusion constructs, e.g. G4-E12+13 and G4-E12, gave variable expression in different experiments. These experiments were repeated several times, and the transactivation data shown represent the activity of similar levels of protein. If we assume that combination of E10 and E11 reconstituted a relatively weak transactivation domain and that combination of E12 and E13 reconstituted a relatively strong transactivation domain, then combination of the two disrupted activation domains, E11 and E12, would not have reconstituted a third domain. Thus, the full activity of transactivation was dispersed among the four exon fragments. These data demonstrate that the integrity of the PST region is crucial for a high level of activation. A minimal deletion in the C-terminal exon (E13) can result in a 50% reduction in transactivation, which may be sufficient to cause aniridia. The residual PST domains retain substantial transactivation function and thereby may cause a relatively mild phenotype (29), although there was no prior evidence that the truncated PAX6 proteins would be stable in the context of the native PAX6 protein.

**Self-squelching Effect of the PST Domain**—To test the hypothesis that the PST domain activated transcription by protein-protein interactions with coactivators, we tested whether a PST peptide that lacked a DNA-binding domain could inhibit the transactivation function of a PST peptide attached to the GAL4-DBD. The coding sequence of the PST domain was inserted into the pRC-CMV expression plasmid and fused to an N-terminal Flag epitope tag. The reporter plasmid we used, PIGC-luc, had five copies of GAL4 binding sites inserted in the
upstream regulatory region of the luciferase gene. The GAL4-PST hybrid activator stimulated efficient transcription of the PlGC-luc reporter in HeLa cells in transient transfection assays. The Flag-PST peptide inhibited the transactivation activity of GAL4-PST in a dosage-dependent manner (Fig. 1C), suggesting that the overexpressed Flag-PST peptide could compete with the GAL4-PST activator for coactivators that were required for activation. It is possible that multiple coactivators may interact with each exon fragment of the PST domain. These coactivators act synergistically to stimulate transcription. This hypothesis is consistent with our data showing that any combination of two adjacent exons fused to the GAL4 DNA-binding domain could produce a moderate level of transcription. Any small deletions may reduce the size of the domain’s interacting surface with coactivator(s) and thus affect the synergism.

DNA Binding and Transactivation by PAX6 Mutants—We next tested whether the incomplete PST domains, which have been shown to retain residual transactivation function when fused to the GAL4-DBD, also exhibit similar activities within the context of native PAX6 protein. First, wild-type and mutant PAX6 expression vectors were generated by inserting PAX6 cDNAs downstream of the cytomegalovirus (CMV) promoter in the pRc-CMV expression vector (Fig. 2). The expression vectors were transfected into HeLa cells with a reporter plasmid, pG5EC-P6CON-CAT, which contains six copies of the PAX6 PD binding sites (P6CON) inserted upstream of the E1B TATA box and the CAT gene (45). The wild-type PAX6 stimulated efficient transcription of the pG5EC-P6CON-CAT reporter (Fig. 3A) but not the control reporter pG5EC-CAT that lacked the P6CON binding sites. Deletion of the C-terminal exon (E13) resulted in a more than 50% reduction in transactivation. Further deletion of E12 caused another 20% loss of activity. Deletion of more exons caused gradual loss of the remaining activity. These results are very similar to those obtained in the GAL4-PST fusion experiments, except that here E10 also showed slight activity. The different levels of transactivation of the mutants were not as dramatic as those observed in the
other two mutants (PAX6-(1–306) and PAX6-(1–272)) were deletions comparable with those of the wild-type PAX6. Although the PD of PAX6 (aa 1–127) (Fig. 3B) or other endogenous activators binding the P6CON sites.

Interestingly, PAX6 mutants with more than two exons deleted appeared to repress this putative endogenous factor activity, possibly because overexpressed mutant PAX6 could compete for the P6CON binding sites with the endogenous factor. To test this possibility, we analyzed the DNA binding properties of the wild-type and mutant PAX6 proteins, using nuclear extracts prepared from HeLa cells that were transfected under the same experimental conditions as used for transactivation assays were assayed by Western blotting using a polyclonal antibody directed against the wild-type and mutant PAX6, nuclear extracts prepared from NIH 3T3 cells that were transfected under the same experimental conditions as used for luciferase assays. Wild-type and mutant PAX6 proteins with truncated PST domains were stable and fully functional in DNA binding, suggesting that they may act as potential repressors of the wild-type PAX6 protein by competing for common DNA-binding sites.

Transactivation of PAX6 Mutants with Homeodomain DNA-binding Sites—The wild-type PAX6 protein activated transcription through DNA binding of either the PD or the HD. To determine whether the truncated PST domains produce similar patterns of transactivation through DNA binding of the HD, as through DNA-binding of the PD, we tested a luciferase reporter plasmid, P3luc, in which the three copies of CD19–2-(A-ins) binding sites in the CD19-luc reporter were replaced by one copy of the P3 sequence containing a HD binding site (21). In NIH 3T3 cells, the wild-type PAX6 activated the P3luc reporter expression nearly 20-fold above the basal level (Fig. 3D, RC-CMV). Deletion of E13 resulted in about 70% reduction in activation. Deletion of additional exons resulted in a gradual loss of activation (Fig. 3D). To verify the protein expression of the wild-type and mutant PAX6, nuclear extracts prepared from cells that were transfected under the same experimental conditions as used for transactivation assays were assayed by Western blotting using a polyclonal antibody directed against the wild-type PAX6 (aa 1–127) (Fig. 3E). Three of the four mutants (PAX6-(1–395), PAX6-(1–344), and PAX6-(1–306)) showed protein levels comparable with those of the wild-type PAX6. These findings resemble those obtained from assays using PD binding sites (Figs. 3A and 4). Thus, the PST domain produced a similar transactivation pattern regardless of which DNA-binding domain was used.

Partial Activity of Mutant PAX6 with Truncated PST Domains—Since the pG5SEC-P6CON-CAT reporter detected a high basal activity that made the assay of mutant PAX6 activity less sensitive, we tested another reporter plasmid, CD19-luc, which contained a luciferase reporter gene driven by a basal promoter and three copies of the PD binding site CD19–2-(A-ins) in the upstream regulatory region (21). We also switched to NIH 3T3 cells, since the transfection recipient cells (NIH 3T3 cells) do not express endogenous PAX6 protein (Fig. 6A). As predicted, CD19-luc produced a low basal activity comparable with the activity produced by the mutant PAX6-(1–270) that lacked the PST domain (Fig. 4). The wild-type PAX6 protein activated the transcription of this reporter to 10-fold above basal levels. In contrast, mutant PAX6-(1–395), with E13 deleted, lost nearly 70% of the wild-type activity. Mutant PAX6-(1–344), with E12 and E13 deleted, showed a greater than 80% loss of activity. Mutant PAX6-(1–306), with three exons deleted, showed a reduction in activity to near basal levels (Fig. 4). The level of protein expressed and its binding to the CD19–2-(A-ins) sites was assessed by Western blotting and EMSA, respectively (Fig. 6, A and B), using nuclear extracts prepared from NIH 3T3 cells that were transfected under the same conditions as used for luciferase assays. Wild-type and mutant PAX6 proteins of expected sizes were detected at similar levels of expression with no detectable endogenous PAX6 protein (Fig. 6A). Both the wild-type and mutant PAX6 proteins bound to the CD19–2-(A-ins) probe (Fig. 6B), thus mini-
FIG. 3. Transactivation function of wild-type PAX6 and mutant PAX6 with truncated PST domains. A, wild-type and mutant PAX6 were expressed in HeLa cells by transient transfection and then tested for their ability to activate transcription of the CAT reporter gene, which was linked to a composite promoter containing six copies of the PAX6 PD binding sites. Schematic diagrams of wild-type and mutant PAX6 constructs are described in the legend to Fig. 2. CAT activity is shown relative to that of the full-length PAX6, which is given as 100. Results of CAT assays are expressed as mean ± S.E. of duplicate assays from a representative cotransfection experiment. Deletion of the last exon in the PST domain dramatically reduced transcriptional activation. Deletions of more exons in the PST domain caused a gradual loss of activation. The slight activation (≈ 20% of activity) of the P6CON CAT reporter by the Rc-CMV vector is probably due to the low amount of endogenous PAX6 or other activators in HeLa cells. Black bars indicate reporter containing the P6CON PD binding sites. Hatched bars indicate reporter without P6CON binding sites. B, protein expression of transfected wild-type and mutant PAX6 expression constructs in HeLa cells. Nuclear extracts prepared from HeLa cells transfected under the same experimental conditions as used for CAT assays in Fig. 3A were subjected to Western blot analysis. Proteins were resolved by SDS-10% PAGE, and the wild-type and mutant PAX6 proteins were detected by enhanced chemiluminescence using a polyclonal antibody.
Transactivation Function of PAX6

Deletion mutants in NIH 3T3 cells. Schematic diagrams of the internal deletion constructs are described in the legend to Fig. 2. Mutant and wild-type PAX6 proteins were expressed in NIH 3T3 cells by transient transfection and examined for their ability to activate transcription of the CD19-luc reporter that bears the PD binding site CD19–2 (A-ins) (21). The relative activities of luciferase are shown as mean ± S.E. of three separate transfection experiments.

2-(A-ins) (21). The relative activities of luciferase are shown as mean ± S.E. of three separate transfection experiments.

Ammonizing the possibility of reduced DNA binding of the mutants. Here, the data we obtained using a more sensitive transfection assay confirmed the findings of the pG5EC-P6CON-CAT assay (Fig. 3A) and demonstrated that mutant PAX6 with truncated PST domains had much less residual transactivation activity than predicted from the GAL4 fusion experiments.

Taking these data together with the data from previous experiments, we concluded that the integrity of the PST domain is crucial for high level transactivation of the PAX6 protein. Partial or complete deletions of the PST region reduced the transactivation ability of the resulting mutants but did not affect their DNA binding ability. Deletion of E13 reduced activation by 70%, which may be sufficient to cause aniridia. Whether the remaining 30% of activity of such mutants can contribute to transactivation and result in a less severe phenotype remains a question. Other mutants with more than two exon fragments deleted had only negligible transactivation activity, but such mutants may act as potential repressors to the wild-type PAX6 protein, which may lead to a more severe phenotype.

Synergism of the Four Constituent Exons of the PST Domain—The 70% loss of activity after deletion of E13 prompted us to test whether most transactivation activity resides in the C-terminal exons, i.e. E13. Internal deletion constructs were generated that had the PST region deleted from their N-terminus (Fig. 2). The N-terminal end of the PST domain begins with the 6-aa peptide stretch LRNQRR, which is characterized by 50% basic residues and is identically conserved during evolution from Drosophila to human but has an unknown function. A mutant PAX6-LGNQQG was generated by substituting the three conserved arginines with three glycines using site-directed mutagenesis. This mutant activated the transcription of the CD19-luc reporter in NIH 3T3 cells to nearly the same level as did the wild-type PAX6 protein in transient cotransfection assays (data not shown).

For convenience in cloning, we created a SacII restriction site near the junction of the HD and the PST domain by site-directed mutagenesis. The SacII restriction site was inserted downstream of this highly conserved peptide stretch to minimize any potential effects on DNA binding of the HD. After an in frame insertion of the SacII site, two extra amino acids (Pro and Ala) were generated between aa 277 (Gln) and aa 278 (Ala) immediately downstream of the peptide stretch LRNQRR (aa 271–276; Fig. 7B). The PAX6-SacII construct was transfected with the CD19-luc reporter into NIH 3T3 cells and produced nearly 90% of the wild-type PAX6 activity (Fig. 4). We then tested the internal deletion mutants in which one exon was deleted at a time from the N terminus of the PST region. Mutant PAX6-E11+12+13 with deletion of E10 lost 75% of the wild-type PAX6 activity and 72% of the PAX6-SacII activity. Mutant PAX6-E12+13, with deletion of exons 10 and 11, showed a greater than 80% loss of the wild-type activity. Mutant PAX6-E13, with deletion of three exons, showed a reduction in activity to basal levels. Mutant PAX6-(307–359 +3), with a PST domain encompassing E11 and one-third of E12, followed by three out-of-frame amino acids, did not show transactivation. To test the DNA binding ability of these mutants, we performed EMSAs using nuclear extracts prepared from transfected NIH 3T3 cells. The quality of each transcription was ensured by performing duplicate transfections in which two sets of transfections were used for the luciferase assay and one set was used for preparation of nuclear extracts. The nuclear extracts were used for Western blot analyses (Fig. 6A) using a polyclonal antibody directed against the PD of PAX6 (aa 1–127) as well as for gel shift assays. All of the PST mutants except PAX6-(1–272) were detected at a similar level and showed a strong binding to the CD19–2 (A-ins) probe, as did the wild-type PAX6 (Fig. 6B). Thus, the reduction in transactivation of the mutants was due to the deletion of the PST domain rather than to a reduction in DNA binding. We found that although PAX6-(1–272) binds DNA with higher affinity, its level was consistently lower in all of the experiments shown. To determine if higher expression of PAX6-(1–272) obeyed a dose-response relationship, increasing amounts of PAX6-(1–272) plasmid were used in transient transfection experiments. Western blot analysis revealed that increasing the amount of plasmid

antibody directed against the PAX6 PD (aa 1–127) (44). Each lane contained the same quantity of nuclear extract. The protein levels of mutant PAX6-(1–306) and PAX6-(1–372) were lower than other mutants and wild-type PAX6. A low level of endogenous PAX6 was detected in HeLa cells. The migrating position of the pretransfected protein markers are indicated. C, the DNA-binding function of the PST truncation mutants as determined by EMSA of the same nuclear extracts used in Western analysis in Fig. 3B. The wild-type and mutant PAX6 proteins were tested for their ability to bind the PD binding site, P6CON. Each lane contained the same quantity of nuclear extract. The numbers listed on each lane were taken from the volume reports after quantitation of each shifted band with a PhosphorImager. The two truncation mutants that were expressed at lower levels on Western blotting showed stronger binding than wild-type PAX6. D, transactivation pattern of mutant and wild-type PAX6 with reporters bearing HD binding sites. Mutant and wild-type PAX6 proteins were expressed in NIH 3T3 cells by transient transfection and then tested for their ability to activate transcription of a luciferase reporter gene driven by the P3 HD binding site (21). The relative activities of luciferase are shown as mean ± S.E. of three separate transfection experiments. E, protein expression of transfected wild-type and mutant PAX6 expression constructs in NIH 3T3 cells. Nuclear extracts prepared from NIH 3T3 cells transfected under the same experimental conditions as used for luciferase assays in Fig. 3D were subjected to Western blot analysis. Proteins were resolved by SDS-10% PAGE, and the wild-type and mutant PAX6 proteins were detected by enhanced chemiluminescence using a polyclonal antibody directed against the PAX6 PD (aa 1–127). Each lane contained the same quantity of nuclear extract. Molecular mass standards used are prestained SDS-PAGE standards from Bio-Rad and are expressed in kilodaltons.

3 H. K. Tang, S. Singh, and G. F. Saunders, unpublished observations.
Transactivation Function of PAX6

...was no significant difference in the activity (Fig. 6D).

Taken together, our data demonstrate that the transactivation function of PAX6 is not organized as a single discrete domain localized to the C-terminal exons, e.g., E13, since E13 alone did not produce any transactivation activity and the deletion of an N-terminal exon (E10) reduced the transactivation activity by more than 70%. Nor is the transactivation function localized to the N-terminal exons, e.g., E10, since E10 alone did not produce any transactivation activity and the deletion of a C-terminal exon (E13) also resulted in 70% reduction of transactivation. The transactivation function is not localized in the middle exons either, since deletion from either end resulted in a dramatic reduction in transactivation activity. Moreover, our data cannot be explained simply by a two-subdomain theory whereby a boundary may be drawn between E11 and E12 to form a left subdomain and a right subdomain or a single domain spanning the boundary between exons 11 and 12. Indeed the combination of the two middle exons, the combination of two N-terminal exons, and the combination of the two C-terminal exons all produced similar levels of transactivation (Fig. 2A). Nor did we find any repression domains such as those found in some other Pax genes; all deletions caused dramatic reductions in transactivation activity. Therefore, we conclude that PAX6 contains a large transactivation domain that consists of four exons encompassing the entire PST region in the C terminus of PAX6. The four exon fragments act synergetically to produce the full activity of transactivation.

Mutagenesis of E13—The data presented so far demonstrate that all four exons of the PST region are responsible for the full activity of transactivation. Like other transactivation domains, the PST domain may activate transcription by contacting multiple components of the basal transcription machinery such as TAFs or other coactivators. It is unclear, however, whether the effect of whole-exon deletion in the PST region is caused by a simple reduction in size of the surface that interacts with a particular coactivator or by alteration of the entire structure required for interacting with all coactivators. To determine the role of an individual exon in the transactivation function of the PST domain, we selected E13 for more detailed mutagenesis. This 28-aa exon is highly conserved during evolution in organisms ranging from squid to humans with 79% amino acid identity (Fig. 7B).

First, constructs with small deletions in E13 were generated by removing 9 aa at a time from the C-terminal end and then examined for their activity in transactivating the CD19-luc reporter in NIH 3T3 cells. Surprisingly, mutant PAX6-(1–413), with only 9 aa deleted, showed a more than 50% reduction in transactivation (Fig. 5). This reduction was not due to a loss of DNA binding or lower expression, since the nuclear extracts prepared from the same transfections showed a substantial binding of the mutant protein to the CD19–2-(A-ins) probe and similar expression (Fig. 6, A and B). Mutant PAX6-(1–404), with 18 aa deleted, showed no more than a 50% reduction in wild-type activity. Both mutants showed significant reduction of transactivation, but less than the reduction for mutant PAX6-(1–395) whose entire E13 was deleted (70% reduction). It is unclear whether the C-terminal 9 aa of E13 are involved in interactions with a coactivator or important for the structure of the PST domain. The N-terminal third of E13 is probably responsible for the additional 20% loss of activity of mutant PAX6-(1–395).

Next, we introduced amino acid substitutions into the N-terminal half of E13 to alter the structure of E13. We selected four Val residues in the N-terminal half of E13 that comprises an evolutionarily highly conserved 7-aa stretch, with one conservative substitution of Val by Ile. Hydrophobic residues such as valines and isoleucines are generally involved in building the structural skeletons of proteins rather than in making surface contacts. Secondary structural prediction by the Garnier-Osguthorpe-Robson computer program revealed β-sheet structures of this region (Fig. 7A). The four valine residues were substituted by four glycines (mutant PAX6-(4V/G)) such that the β-sheet structure of this region was abolished according to the computer program prediction. Mutant PAX6-(4V/G) showed only a 25% reduction in wild-type activity. To test whether the 25% reduction in mutant PAX6-(4V/G) activity plus the 50% reduction in the mutant PAX6-(1–413) activity could account for the 70% reduction in mutant PAX6-(1–395) activity, we tested another mutant, PAX6-(4V/G+27), in which the 15 aa following the 4V/G stretch were replaced by 27 unrelated amino acids that resulted from out-of-frame translation due to a 1-base pair deletion mutation. Frame shift mutations are commonly seen in naturally occurring PAX6 mutations. This mutant showed a 60% reduction in transactivation, which is close to the reduction caused by deleting the entire E13. We have also tested a mutant with a single missense mutation, PAX6-S398A, which resulted in less than a 10% reduction in wild-type PAX6 activity (data not shown). The DNA binding function of all of these mutants was unaffected by mutations as shown by EMSA (Fig. 6B). Together, our data suggest that even within one exon, the transactivation function is relatively unlocalized. Indeed regional deletions or multiple amino acid substitutions in E13 did not abolish the activity of the entire exon. Moreover, the data suggest that β-sheet structure might be important for the interaction of E13 with coactivators and that amino acid substitutions result in less dramatic effects than deletions. Interestingly, no missense mutations have been detected in the PST region in aniridia or small eye. Therefore, it is intriguing that the amino acid sequences of the PST region are highly conserved during evolution, while amino acid substitutions have no dramatic effects.

DISCUSSION

The isolation of PAX6 has provided an entry to elucidating the pathogenesis of aniridia and the mechanism of eye organo-

![Fig. 5. Alterations in transcriptional activation activity of PAX6 by mutagenesis in exon 13. Schematic diagrams of the E13 mutation constructs are described in the legend to Fig. 2. Mutant and wild-type PAX6 proteins were expressed in NIH 3T3 cells by transient transfection and examined for their ability to activate transcription of the CD19-luc reporter bearing the PD binding site, CD19–2-(A-ins) (21). The relative activities of luciferase are shown as mean ± S.E. of three separate transfection experiments.](http://www.jbc.org/content/316/5/7218.f5)
Fig. 6. Analysis of expression and DNA-binding function of mutant PAX6. A, Western blot analysis of nuclear extracts prepared from NIH 3T3 cells transfected under the same conditions as used in Figs. 4 and 5. Proteins were resolved on 10% SDS-PAGE. Each lane contained the same amounts of protein. Mutant and wild-type PAX6 proteins were detected by a polyclonal antibody directed against the PAX6 PD (aa 1–127) PAX6-(16–422) using the ECL kit (Pharmacia Amersham Biotech). Molecular mass standards used are prestained SDS-PAGE standards from Bio-Rad and are expressed in kilodaltons. B, EMSA of the ability of mutant and wild-type PAX6 proteins to bind the CD19–2-(A-ins) site, using nuclear extracts that were prepared from one of the triplicate transfection experiments described in the legends to Figs. 4 and 5. Each transfection was performed in triplicate (two sets were used for luciferase assays, and one set was used for preparing nuclear extracts). Each lane contained the same amount of nuclear extract. C, Western blot analysis of nuclear extracts prepared from NIH 3T3 cells transfected with PAX6 or increasing amounts of PAX6-(1–272). Proteins were resolved on 10% SDS-PAGE. Each lane contained the same amount of proteins. Mutant and wild-type PAX6 proteins were detected by a polyclonal antibody directed against the PAX6 PD (aa 1–127) using the ECL kit (Pharmacia Amersham Biotech). D, schematic diagrams of the PAX6 mutation constructs are described in the legend to Fig. 2. PAX6 and increasing concentrations of mutant protein PAX6-(1–272) were expressed in NIH 3T3 cells by transient transfection and examined for their ability to activate transcription of the CD19-luc reporter bearing the PD binding site, CD19–2-(A-ins) (21). The relative activities of luciferase are shown as mean ± S.D. of two separate transfection experiments.
The PAX6 protein is characterized by two DNA-binding domains, a PD, and a HD linked by a Gly/Gln-rich hinge region. The remaining C-terminal 35% of the molecule contains a PST domain. Such a structural organization is found in proteins produced by other Pax genes with dual DNA-binding domains (Pax3, Pax4, and Pax7). Perhaps this structure allows flexibility of the PST region in protein-protein interactions.

Here, we carried out a structure-function analysis of the transactivation function of PAX6. By fusing PAX6 to the heterologous DNA-binding domain of GAL4 (aa 1–97), we demonstrated that the PST region of PAX6 functions as a transactivation domain independent of other regions of PAX6. Dissection of the PST region revealed that the transactivation function is dispersed among the four constituent exons and that the four exons cooperate to produce the full activity of transactivation. No repression domains were found in PAX6, in contrast to those found in the C-terminal region of PAX2, PAX5, and PAX8 (32).

The structural motifs of transcriptional activation domains are poorly understood, but they may include isoleucine-, proline-, glutamine-, serine/threonine-rich regions and acidic regions (47, 48). The PST region of PAX6 encompasses 152 aa with a high proportion of proline (15%), serine (18%), and threonine (11%) residues. Secondary structure analysis by both the Chou-Fasman and the Garnier-Osguthorpe-Robson computer programs predicts that the PST region consists primarily of $\beta$-sheets and turns (Fig. 7A). This agrees with the circular dichroism spectroscopic findings that acidic activators GAL4 and GCN4 adopt $\beta$-sheet structures (49–50).

The most striking feature of the PST region lies in its high evolutionary conservation. The amino acid sequences are 95% identical in vertebrates including humans, mice, quail, and zebrafish (Fig. 7B). Such a high degree of conservation is remarkable, because transactivation domains are rarely conserved among transcription factors of such diverse species. High conservation is often a unique feature of DNA-binding domains of transcription factors.
domains that bind transcription factors to their specific targets. This is seen in Drosophila pax6, which shows 93% amino acid identity to vertebrate Pax6 in the PD and 90% identity in the HD (20). Yet the PST region in Drosophila pax6 is twice as large as in vertebrates, with no significant homology to the vertebrate homologue except for a high proportion of proline (11%), serine (20%), and threonine (6%). The only significant homology observed is that in a stretch of 6 aa (or 7 aa, if the homeodomain is defined as 60 aa) immediately adjacent to the HD (LRNQRR at positions 271–276) (Fig. 7B) this 6–22 aa stretch is identical in all animals. Despite the divergence of the PST domain between Drosophila pax6 and vertebrate Pax6, mammalian Pax6 can restore the function of Drosophila pax6 and induce ectopic eye formation in Drosophila (51).

The phenotypic expressivity of a transcription activator may be dramatically affected by the overall potency of transcriptional activation. Although the mechanism of transcriptional activation is still unclear, recent evidence suggests that transcriptional domains act to enhance the assembly of the preinitiation complex via protein-protein interactions. These involve specific interactions directly with components of the basal transcription apparatus or indirectly via additional transcription activators (52). The overall potency of transcriptional activation results from the combinatorial effects of the DNA binding affinity, the synergistic actions of coactivators within a complex enhancer element, and the strength of the interaction between an activation domain and its “target.” A reduction in the potency of transcriptional activation of Pax genes may lead to a loss-of-function phenotype such as Pax6 mutations in aniridia and Pax3 mutations in Waardenburg syndrome. Conversely, an increase in the potency may lead to a gain-of-function phenotype such as overexpression of the human Pax6 in transgenic mice that have shown an eye phenotype different from small eye (53) and the Pax3 and Pax7 mutations in alveolar rhabdomyosarcoma (54).

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