Pax genes encode transcription factors known to play crucial roles during the development of specific embryonic tissues. In humans and mice, several abnormalities have been linked to deficiencies in Pax gene dosage, indicating that normal development is particularly sensitive to the level of Pax gene expression. Despite these facts, relatively little is known about how these proteins act as transcriptional regulators. In this study we define the transactivation domains of murine Pax-2, an essential factor in kidney organogenesis. Within the COOH terminus of Pax-2, amino acids 279–373 are essential for transactivation. However, this region alone is insufficient for full transactivation when fused to the paired domain alone or to a heterologous DNA binding domain. Mutation or deletion of the conserved octapeptide sequence results in increased transactivation by Pax proteins. The octapeptide-mediated repression is also seen within a heterologous context using the GAL4 DNA binding domain. Thus transactivation by Pax-2 relies upon several regions within the COOH terminus and is down-modulated by the octapeptide element.

Pax genes are defined by a 128-amino acid DNA binding motif, termed the paired domain (PD), which has been conserved in animal species ranging from sea urchin to humans (1, 2) and which was originally identified in the Drosophila gene pair (3). In the mouse, there are at least nine known members of the Pax family which are subdivided into classes largely based upon conservation of amino acids outside the PD (4). The unique spatio-temporal expression pattern of these genes strongly suggested a role in patterning of the early mouse embryo. To underscore this hypothesis, lesions in three different Pax genes, Pax-1, -3 and -6, were found in three naturally occurring mouse mutants; undulated, splatch, and small eye, respectively (5–7). Not surprisingly, each of these genes is expressed in the primordia that give rise to affected tissues in the mutants. Moreover, the abnormalities are found in heterozygotes, whereas homozygotes typically exhibit more severe phenotypes including embryonic lethality (8). Together, these findings have clearly established that the Pax family of transcription factors is essential for the development of diverse tissues and organs during early patterning events in the embryo.

The Pax-2 gene is required for the development of the mammalian urogenital system from the intermediate mesoderm (9, 10), including the specification and proper differentiation of the renal tubular epithelium from uninduced metanephric mesenchyme (11, 12). The murine Pax-2 gene encodes two proteins, Pax-2a and Pax-2b, which differ by 23 amino acids inserted in-frame in Pax-2a (13). Pax-2a, -5, and -8 constitute a subfamily of Pax proteins which possess specifically conserved amino acids within the PD as well as stretches of homology within the COOH terminus. Among these are a conserved octapeptide, a region rich in proline, serine, threonine, and tyrosine residues (PSTV), and the first two helices of the paired type homeomain. Experiments with Pax-5 have shown that the truncated homeodomain does not bind DNA (14) and that DNA recognition is mediated exclusively by the PD, which consists of NH2- and COOH-terminal subdomains, each capable of binding distinct regions within recognition sequences in vitro (15). DNA recognition sequences for the PD of the Pax-2, -5, and -8 subfamily have been described (15–18), and a number of genes have now known to be positively and negatively regulated by Pax-5 (14, 15) and Pax-8 (19, 20). Apart from DNA binding properties, however, relatively little is known about the biochemistry of Pax protein transcription.

To understand how the Pax-2 protein acts as a transcription regulator, we examined the COOH-terminal regions of Pax-2, which confer transcriptional activation or repression. Extensive deletion and mutation analysis was used to delineate domains that are responsible for transcriptional regulatory properties of Pax-2 in transfected cells. Full activation required multiple COOH-terminal regions that were essential but not sufficient by themselves. Surprisingly, the octapeptide sequence acts as a repressor of activation potential, both within the context of the Pax-2 PD and the heterologous GAL4 DNA binding domain.

**MATERIALS AND METHODS**

Plasmids—Pax-2 expression plasmids were engineered to contain a COOH-terminal hemagglutinin epitope using the double-stranded oligonucleotide 5’-CCCAAAGTTGGTGGTATACCCATATGATGTACCAGACTATGCGTACCCG-3’ containing unique BstXI, NdeI, and KpnI sites (underlined sequences, respectively; stop codon shown in bold). The oligomer was digested with BstXI and KpnI and inserted into the corresponding sites of pCMVpax2a (11) to produce p1–95HA (numbering refers to the Pax-2 amino acids encoded by the plasmid). Specific oligonucleotide primer pairs, incorporating a 5’ BstXI site or a 3’ NdeI site, were used in polymerase chain reaction to amplify fragments encoding amino acids 96–144 and 96–204 which were inserted into p1–95HA to generate p1–144HA and p1–204HA, respectively. Full-length Pax-2a and -2b, as well as the COOH-terminal and internal deletions shown in Fig. 1, were constructed by a similar methodology using polymerase chain reaction-amplified products that were inserted into the unique NdeI site in p1–144HA or p1–204HA. To create the PD deletion, p1–415HA was digested with BspEI and FseI, blunt ended with T4 DNA polymerase, and religated. p1–329–415HA, lacking amino acids 330–348, was constructed by cutting with AgeI and blunt ending with Klenow enzyme followed by limited treatment with mung bean nuclease and addition of an XbaI linker. In the case of pCMVpax2bOCT-HA, the plasmid was digested further with XbaI, filled in with Klenow enzyme, and religated to

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‡ The abbreviations used are: PD, paired domain; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus.
FIG. 1. Multiple regions of the COOH terminus contribute to Pax-2 transcriptional activation. Recombinant Pax-2 proteins are depicted schematically and designated according to the amino acid residues encoded by each construct. Panel A, COOH-terminal deletions. Panel B, internal deletions. Full-length Pax-2 proteins are designated 1–415, and a colony is used to represent the junction generated by internal deletions, except for the PD deletion, which is missing amino acids 33–138. Transactivation activity of each is shown to the right. The PD and conserved octapeptide (O) are boxed, and the region rich in proline, serine, threonine, and tyrosine residues is marked -PSTY-. Also shown is the alternative splicing pattern used to generate Pax-2b which is lacking exon 5 and 23.

Transient Transactivation Assays—Cells used in these studies were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and transfected using lipofectaminereagent as described by the manufacturer (Life Technologies, Inc.). For roughly 5 × 105 cells, 1.0 µg of CAT reporter plasmid was cotransfected with 4.0 µg of effector plasmid and 1.0 µg of pCH110 (Pharmacia), a β-galactosidase reporter plasmid used to monitor transfection efficiency. After 48–72 h of transfection, cells were harvested, and extracts were prepared for CAT analysis essentially as described (25). Percent acetylation was determined by scintillation counting, normalized for transfection efficiency, and a value of 1.0 was arbitrarily assigned to control parental plasmids.

RESULTS

To delineate the domains responsible for the transcriptional activation of Pax-2, we first generated a set of COOH-terminal terminal deletions depicted in Fig. 1A. A hemagglutinin (HA) epitope was incorporated at the COOH terminus of each con-
struct to facilitate isolation and identification of the recombi-


The transactivation potential was then assessed in transient transfections using a CAT reporter construct that contained five tandem copies of a Pax-2 binding site, PRS4 (16), upstream of a minimal promoter. A summary of the data obtained from experiments performed in P19 cells is presented in Fig. 1A. Similar results were obtained with NIH 3T3 and 293 cells (data not shown). Full-length Pax-2 proteins, FLAG-epitope tagged proteins are shown as well as the octapeptide substitution mutants. C corresponds to extract from control cells transfected with pCB6+. Panel D, COOH-terminal deletions and octapeptide substitution mutants. Equal amounts of extracts (~5 μg) were combined with Pax-2 binding site probe, H2A-2.2, and binding reactions were separated on native polyacrylamide gels. DNA-protein complexes are shown, and asterisks denote PD-containing complexes generated from proteolytic breakdown of the full-length proteins. Panel E, internal deletions. Flag-tagged Pax-2a is also shown (FL1–415a). Experiments shown in panels A–D were performed with extracts from NIH 3T3 cells and that in panel E from 293 cells. Equivalent results were achieved with either cell line for all of the constructs tested.

To verify that these proteins were properly expressed, Western blot analyses were performed on extracts from transiently transfected cells. Fig. 2, A–C, shows typical results obtained using an anti-Pax-2 antiserum. The Pax-2 antiserum was raised against a COOH-terminal fragment and therefore does not recognize Pax-2 proteins truncated after amino acid 204. It also reacted weakly with proteins containing only amino acids
of transactivation are utilized by these classes of Pax proteins. A slight variation was observed in protein expression levels, this did not correlate with the transactivation activity. In fact, many of the deletion mutants showed equal or greater amounts of Pax-2 protein compared with wild type Pax-2a or Pax-2b. Thus, the reduced levels of transactivation observed with the mutants are not due to increased protein instability. The results also illustrate that the 23 amino acids encoded by the additional exon in Pax-2a are unlikely to play a major role in transactivation.

The site-specific DNA binding activity of these proteins was assessed in gel mobility shift assays, and none of the proteins tested exhibited reduced DNA binding activity, except for the deletion just after the PD (1–144). Typical results from assays using the H2A-2.2 binding site (14) for COOH-terminal and internal deletions are shown in Fig. 2, D and E, respectively. Identical results were obtained using the PRS4 binding site which is present in the reporter plasmid used to assay Pax-2 transactivation (data not shown). DNA–protein complexes were formed efficiently in each case, and the specificity of these complexes was verified by competition and antibody supershift experiments (data not shown). Many of the proteins defective in transactivation bound DNA to an extent equal to or greater than that of the wild type Pax-2 proteins, further illustrating that reductions in transactivation were not due to reduced protein levels. The faster migrating complexes found with full-length and COOH-terminal deletions of Pax-2 were determined to be the result of proteolytic cleavage after the PD and near amino acid 200 which occur during the extraction procedure. Note that the DNA–protein complexes marked with an asterisk (Fig. 2D) do not change in mobility as the COOH-terminal deletions increase and that the 1–204 protein (Fig. 2E) migrates at that same position. In addition, we have examined the localization of these proteins by indirect immunofluorescence and confirmed their nuclear localization (data not shown). With one exception, these data show that the deficiencies in transactivation by mutant Pax-2 proteins were not due to improper expression nor their failure to bind target DNA.

Since the transactivation activity increased in deletion mutants that spanned the conserved octapeptide we made substitution mutants by replacing the eight wild type amino acids in this element with unrelated amino acids. Experiments performed with these octapeptide mutants showed a 2–3-fold increase in transactivation compared with wild type Pax-2 (Fig. 3A), which was similar to the increase observed for deletions covering the octapeptide region. These results demonstrate that the elimination of the octapeptide sequence was responsible for the increase in Pax-2 transactivation and suggest that this region acts to down-modulate Pax-2 activity. To test this possibility further, the octapeptide region in Pax-8, which is closely related to Pax-2 and recognizes similar DNA target sequences, was deleted. When tested in transient transfection assays, the activation potential of Pax-8 was also elevated 2–3-fold in the deleted version (Fig. 3A). The increase in transactivation was also demonstrated by using increasing amounts of effector plasmids (Fig. 3B), indicating that these effects were dose-dependent. This experiment also showed that Pax-2 and Pax-8 did not exhibit a decrease in transactivation potential at high effector concentrations, which has been observed for Pax-6 (2) and Pax-3 (26), and may indicate that different mechanisms of transactivation are utilized by these classes of Pax proteins.

To verify the assignment of transactivation domains made by the experiments described above, chimeras were generated between the heterologous DNA binding domain of GAL4 and various portions of the Pax-2 COOH terminus. A diagram of the chimeric constructs and a summary of the results from these experiments are presented in Fig. 4. A region spanning amino acids 197–415 of Pax-2 was capable of activating transcription 48–61-fold over that of the GAL4 DNA binding domain alone. In comparison, a similar region from human PAX-6 which was previously shown to act as an independent transactivation domain (22) stimulated transcription 15-fold, whereas an activation domain from GAL4 (21) gave nearly 57-fold activation in these studies. Thus the COOH terminus of Pax-2 contains a strong transactivation domain that can function on a heterologous DNA binding domain. Deletions made within the Pax-2 COOH-terminal fragment sharply reduce transactivation potential. For example, GAL4:278–415 and GAL4:197–373a possess 8-fold and 27-fold activity, respectively, underscoring the importance of residues flanking amino acids 278–373.

Several of the GAL4 chimeras were then used to test the effect of reintroducing the octapeptide sequence. Addition of the octapeptide to two different Pax-2 chimeras, GAL4:278–415 and GAL4:197–415b, resulted in a roughly 2–4-fold decrease in transactivation (Fig. 4). As a control, an unrelated stretch of amino acids placed within the same position showed no reduction in transactivation. Activation by a chimera bearing two
octapeptides act as transcriptional repressor domains. How this small element functions is presently unknown, although its ability to repress within a heterologous context suggests that it acts as an independent domain. The presence or absence of the octapeptide did not correlate with protein levels observed in this study (see for example Fig. 2C). Chalepakis et al. (28) observed dimerization of truncated Pax-3 proteins and suggested that sequences around the octapeptide may mediate dimer formation. These Pax-3 dimers were dependent in part on the alignment of half-sites in the DNA recognition sequence and thus may be mediated by DNA binding to specific sequences that allow association of two proteins on the DNA target. No evidence of dimerization was observed with full-length Pax-3 proteins (28). In our experiments, Pax-2 bound as a monomer to a single DNA site (H2A-2.2). This is evident by the single protein-DNA complex when intact, full-length Pax-2 is bound (for example, see Fig. 2E) and agrees with previous studies using Pax-2 (13, 16, 18). Furthermore, mixing experiments using full-length and truncated Pax-2 bound to H2A-2.2 do not show any intermediate complexes indicative of heterodimer formation (data not shown).

The dosage effect of the octapeptide sequence, resulting in nearly 20-fold repression with two copies, and the fact that a similar insertion of unrelated amino acids does not alter transactivation support the view that the downstream transactivation domains are not disrupted by the octapeptide. This element could affect the way in which Pax proteins interact with other factors involved in transcription, and this may determine the efficiency of transactivation. The octapeptide element appears to act in cis in that we have failed to find repression of transactivation in trans by the octapeptide in cotransfection experiments. This indicates that its function is specific to the protein in which it is contained. Down-modulation of Pax transcriptional activation in vivo by the octapeptide could conceivably establish a threshold of Pax function which is acutely affected by gene dosage or haploinsufficiency.

Since multiple COOH-terminal domains contribute to transcriptional activity it is unlikely that single amino acid substitutions would completely inactivate Pax-2 and lead to a true loss of function. Indeed, the majority of Pax mutations described to date eliminate the DNA binding activity of the PD or result in large deletions of the COOH terminus which is consistent with this hypothesis (5–7, 28–30). One example for Pax-2 has recently been described in which a family displaying renal abnormalities carries a mutation leading to a truncated protein with an altered octapeptide (10). It is possible that point mutations in the COOH terminus have subtle consequences on Pax-2 activity which cannot be easily detected phenotypically. On the other hand, the possibility that a specific mutation in the COOH terminus could completely inactivate Pax-2 cannot be ruled out, and further characterization of naturally occurring mutations in Pax-2 and other Pax genes will help to clarify this question. Moreover, it should be kept in mind that our studies utilized an artificial Pax-2 reporter, and it will be important to evaluate the activity of Pax-2 mutants on endogenous targets of Pax-2 regulation, such as the recently identified candidates p53 (31) and En-2 (32).

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Fig. 4. Transcriptional regulatory domains of Pax-2 function on a heterologous DNA binding domain. GAL4-Pax-2 chimeras are depicted schematically to the left, and transcriptional activation activity for each is shown to the right. The GAL4 DNA binding domain (BXG) is shown as a filled cross-hatched box, and GAL4 activation domain II (21) is open cross-hatched. The activation domain from Pax-6 is hatched. Portions of Pax-2 fused the GAL4 DNA binding domain are shown as open boxes, and numbers to the left indicate the amino acids, which are encoded. A gray box denotes the wild type Pax-2 octapeptide sequence (+ OCT: Gly-Ser-Tyr-Ser-Ile-Asn-Gly-Ile-Leu-Gly-Ile) and a checkered box is the unrelated control sequence (−OCT: Asp-Pro-Val-Asp-Gly-Val-Gly-Ser) generated from antisense orientation of the insert. P19 cells were cotransfected with GAL4 chimera expression constructs (4 μg) and a CAT reporter construct (1 μg) containing five copies of the GAL4 upstream activating sequence and assayed for CAT activity 48 h later. The values shown for fold activation are mean values normalized to the unfused GAL DNA binding domain (BXG), which was assigned a value of 1.0.

Copies of the octapeptide were reduced almost 20-fold, dramatically demonstrating that the level of repression correlated with the number of octapeptide sequences. To examine the effect of the octapeptide in another context we introduced the Pax-2 octapeptide sequence between the GAL4 DNA binding domain and the Pax-6 transactivation domain. The octapeptide reduced transactivation from 15- to 2.2-fold in the Pax-6 chimera. Note that the Pax-6 protein does not normally contain the octapeptide. These results suggest that the octapeptide can act as a repressor domain and that its effects are additive.

DISCUSSION
In this report, we have utilized a series of deletion mutants to define the regions of Pax-2 which underlie its transcriptional regulatory properties. We find that full transactivation by Pax-2 requires a COOH-terminal fragment spanning amino acid 349-415 which encompasses the parallel homeodomain and the PSTM region. A smaller region within this fragment, namely amino acids 350-373, appears to be essential for transactivation activity. This result is consistent with findings from a previous report which showed that a Pax-2 protein truncated at amino acid 349 was unable to activate transcription (16). Studies of Pax-3 (26), Pax-6 (3, 22), and Pax-8 (27) have also found that portions of the COOH terminus are required for transactivation, and each of these regions is characterized rich in proline, serine, and threonine residues. Examination of the primary amino acid sequence in these regions shows no clear stretches of homology apart from some short sequences conserved between Pax-2 and Pax-8 which do not possess any obvious structural or functional motifs. Nevertheless, the results demonstrate that multiple regions are essential for full transactivation and suggest that several domains act in concert either through conformational effects or by additive interactions with other proteins.

This report also demonstrates that the Pax-2 and Pax-8
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