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The Oct-2 Glutamine-Rich and Proline-Rich Activation Domains Can Synergize with Each Other or Duplicates of Themselves To Activate Transcription

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The B-cell POU homeodomain protein Oct-2 contains two transcriptional activation domains, one N terminal and the other C terminal of the central DNA-binding POU domain. The synergistic action of these two activation domains makes Oct-2 a more potent activator of mRNA promoters than the related broadly expressed octamer motif-binding protein Oct-1, which contains an N-terminal but not a C-terminal Oct-2-like activation domain. Both Oct-2 mRNA promoter activation domains were delineated by truncation analysis: the N-terminal Q domain is a 66-amino-acid region rich in glutamines, and the C-terminal P domain is a 42-amino-acid region rich in prolines. The Q and P domains synergized with each other or duplicates of themselves, independently of their N-terminal or C-terminal position relative to the POU domain. The C-terminal P domain, which differentiates Oct-2 from Oct-1, also activated transcription in conjunction with the heterologous GAL4 DNA-binding domain. Oct-2 thus contains three modular functional units, the DNA-binding POU domain and the two P and Q activation domains. An electrophoretic mobility shift assay with a variety of these Oct-2 activators revealed a distinct complex called QA that was dependent on the presence of an active glutamine-rich activation domain and migrated more slowly than the Oct-2–DNA complexes. Formation of the QA complex is consistent with interaction of the glutamine-rich activation domains with a regulatory protein important for the process of transcriptional activation.

Eukaryotic transcriptional activator proteins are surprisingly flexible and modular in structure, often containing independent regions responsible for binding DNA and activating transcription (reviewed in reference 21). These regions or domains can be exchanged between transcriptional regulatory proteins without loss of function. In such chimeric activators, the promoter specificity of activation generally follows that of the DNA-binding domain, which tethers the activation domain to the appropriate target promoter. Rarely do activation domains discriminate among different promoters, once brought to the promoter by the DNA-binding domain. An exception is the promoter-selective activation domains within the mammalian activators Oct-1 and Oct-2 (32).

Oct-1 and Oct-2 are POU homeodomain proteins that recognize the same sequence, the octamer motif ATGCA AAT, but are differentially expressed in vivo: Oct-1 is broadly expressed, whereas Oct-2 is expressed in a limited number of cell types, including B lymphocytes (reviewed in references 13 and 23). The octamer motif is important for activation of promoters that display the same broad expression pattern as Oct-1, such as the small nuclear RNA promoters and some mRNA promoters, such as that of the histone H2B gene. The octamer motif is also important for activation of promoters, such as those of the immunoglobulin genes, whose activities are restricted to B cells, which generally express Oct-2 in addition to Oct-1.

This cell specificity of promoter activation by the octamer motif can be reproduced when the octamer motif is separated from its natural promoter context and linked to a heterologous mRNA promoter. Thus, the octamer motif directs expression of typical mRNA promoters in B cells (7, 9, 11, 28, 34) and a small nuclear RNA promoter in both B cells and non-B cells (28). The differential response of these promoters to the octamer motif can be explained by the preferential ability of activation domains within the broadly expressed Oct-1 protein to activate a small nuclear RNA promoter and of activation domains within the cell-specifically expressed Oct-2 protein to activate an mRNA promoter (32).

mRNA promoter activation domains in Oct-2 have been mapped in the non-B-cell line HeLa, which does not normally express Oct-2 but in which overexpression of Oct-2 can lead to activation of transcription from an otherwise B-cell-specific octamer motif-containing mRNA promoter (18). These analyses led to the identification of two separate activation domains, one N terminal and the other C terminal to the central DNA-binding POU domain (10, 19, 29). The N-terminal sequences contain a region rich in glutamine residues, which is reminiscent of the Sp1 glutamine-rich activation domains (6), and the C-terminal sequences contain a region rich in proline residues, which is reminiscent of the proline-rich activation domain of CTF/NF-I (17).

When assayed in a truncated β-globin promoter containing six tandem copies of the simian virus 40 (SV40) enhancer octamer motif positioned just upstream of the TATA box, both N- and C-terminal activation domains display considerable interdependence, both being required to activate transcription effectively (29). In this same assay, overexpressed Oct-1 fails to activate transcription effectively because its C-terminal region is not active (29). The N-terminal sequences in Oct-1, however, which, like those of Oct-2, contain regions rich in glutamine residues, can functionally replace the N-terminal sequences in Oct-2. Thus, in this instance, the promoter specificity of transcriptional activation is conferred by the number and perhaps the quality of activation domains.

Here, we have delineated the Oct-2 activation domains by truncation analysis. The N-terminal domain is a 66-amino-acid-long glutamine-rich segment, and the C-terminal domain

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TABLE 1. Structures of effectors[a]

Effector	Structure
Oct-2	MASR(1–479)
^{N}Oct-2	MAS(N)(1–479)
Oct-2ΔN^{1}	MASR(95–479)
Oct-2ΔN^{3}	MASR(95–160)PRVA(195–479)
Oct-2ΔN^{4}	MASRP(95–160)PRVA(195–479)
Oct-2ΔN^{5}	MASS(112–160)PRVA(195–479)
Oct-2ΔN^{6}	MASRVA(195–479)
Oct-2ΔC^{2}	MASR(1–357)LV
^{N}Oct-2ΔC^{3}	MAS(N)(1–357)(381–479)
^{N}Oct-2ΔC^{4}	MAS(N)(1–357)(405–479)
^{N}Oct-2ΔC^{5}	MAS(N)(1–357)(427–479)
^{N}Oct-2ΔC^{6}	MAS(N)(1–357)(427–467)
Oct-2ΔC^{7}	MASR(1–357)(438–479)
Oct-2ΔC^{8}	MASR(1–357)(438–479)P
Oct-2ΔC^{9}	MASR(1–357)(450–479)
Oct-2ΔN^{6}ΔC^{2}	MASRVA(195–357)LV
Oct-Q.POU.P	MASRP(95–160)PRVA(195–357)(438–479)P
Oct-Q.POU.Δ	MASRP(95–160)PRVA(195–357)LV
Oct-Δ.POU.P	MASRVA(195–357)(438–479)P
Oct-P.POU.Q	MASR(438–479)PPRVA(195–357)P(95–160)
Oct-Δ.POU.Q	MASRVA(195–357)P(95–160)
Oct-P.POU.Δ	MASR(438–479)PPRVA(195–357)LV
Oct-Q.POU.Q	MASRP(95–160)PRVA(195–357)P(95–160)
Oct-P.POU.P	MASR(438–479)PPRVA(195–357)(438–479)P
Oct-Δ.POU.Δ	Same as Oct-2ΔN^{6}ΔC^{2}
GAL4(1–94)	MASR(GAL4)LPGSRP
GAL4(1–94)1×Q	MASR(GAL4)LPGSRP(95–160)
GAL4(1–94)2×Q	MASR(GAL4)LPGSRP(95–160)(95–160)
GAL4(1–94)1×P	MASR(GAL4)LPGSR(438–479)P
GAL4(1–94)2×P	MASR(GAL4)LPGSR(438–479)P(438–479)P

[a] The numbers in parentheses indicate the contiguous regions of Oct-2 that are present in the effector proteins by the amino acid positions (inclusive). N, presence of a short peptide epitope (SYPYDVPDYASLGGPSR) from influenza virus hemagglutinin protein; (GAL4), presence of the GAL4 DNA-binding domain, which contains positions 1 to 94 in the GAL4 amino acid sequence. Additional sequences are indicated in the single-letter code for amino acids.

is a 42-amino-acid-long proline-rich segment. These two structurally distinct activation domains, called Q and P, respectively, display considerable flexibility: they can synergize with each other or with duplicates of themselves to activate an mRNA promoter effectively, and their precise position with respect to the DNA-binding POU domain is not important. These results reveal that the Oct-2 transcription factor consists of distinct modular units that can be mixed and matched to activate transcription synergistically. During the course of these experiments, we identified a specific association of a possible regulatory factor with the glutamine-rich activation domain, which may be important for transcriptional activation.

MATERIALS AND METHODS

Effector constructs. The empty expression vectors pCG and pCGN, the latter of which encodes an epitope tag, and the pCG and pCGN *oct*-2, *oct*-2ΔN^{1}, and *oct*-2ΔC^{2} derivatives have been described previously (29). The primary structure of each effector protein described here is shown in Table 1. In all cases, the Oct-2 nucleotide and amino acid numbering systems used here are those of Clerc et al. (5). The entire N-terminal truncation ΔN^{6} was introduced by replacing the N-terminal residues up to amino acid 194 (inclusive) by the short amino acid sequence SRVA, encoded by the DNA sequence TCTAGAGTCGCC, in which an *Xba*I recognition sequence (underlined) is compatible with an *Xba*I site that lies just downstream of the translation initiation codon in the pCG series of expression vectors. The pCG*oct*-2ΔN^{3} construct was prepared by inserting a blunt-ended fragment generated by digesting the *oct*-2 cDNA with *Hin*cII (nucleotide 345) and *Sma*I (nucleotide 545) into the unique *Xba*I site in pCG*oct*-2ΔN^{6}. The construct pCG*oct*-2ΔN^{5} was derived from pCG*oct*-2ΔN^{3} by creating an *Nhe*I recognition site immediately N terminal to Oct-2 amino acid position 108 and then fusing the *Nhe*I site to the N-terminal pCG *Xba*I site.

The Oct-2 C-terminal truncation constructs pCG*oct*-2ΔC^{3}, pCG*oct*-2ΔC^{4}, and pCG*oct*-2ΔC^{5} were derived from pCG*oct*-2 by deleting the regions between a blunt-ended *Pst*I site (nucleotide 1137) and blunt-ended *Sty*I (nucleotide 1206), *Dde*I (nucleotide 1278), and *Hha*I (nucleotide 1342) sites, respectively. The 12-amino-acid C-terminal truncation in pCG*oct*-2ΔC^{6} was created by introducing a termination codon by oligonucleotide mutagenesis of pCG*oct*-2ΔC^{5}. The deletions in pCG*oct*-2ΔC^{7} (nucleotides 1138 to 1377) and pCG*oct*-2ΔC^{9} (nucleotides 1138 to 1413) were made by PCR, and both contain a silent C to G transversion at position 1137.

The POU domain expression plasmids that contain the N-terminal 66-amino-acid Q domain and/or C-terminal 42-amino-acid P domain were generated as follows. The fragment that contains the Q domain sequence was generated by PCR with 5′ and 3′ oligonucleotide primers containing the sequences CTGAAGACTGACCAACGGGCAGCCAGCTA and CTGAAGACTGTGGTTGGGAGGTCAGAA, respectively. The 5′ and 3′ primers anneal to *oct*-2 nucleotides 349 to 363 and 530 to 542, respectively. Note that both primers contain *Bbs*I recognition sites (underlined), and this enzyme is particularly well suited for engineering recombinant proteins because it creates a 4-base 5′ overhang at any sequence positioned 2 bp away from the *Bbs*I recognition site, so that the recognition site itself does not have to be a part of the cleaved fragment. *Bbs*I cleavage of the amplified product leaves ACCA and TGGT 5′ overhangs (boldface) without the *Bbs*I recognition site. The P domain-containing fragment was similarly prepared by PCR with 5′ CTGAAGACTTACCACCCCCGG CCACC (nucleotides 1378 to 1392) and 3′ CTGAAGACTA TGGTGGCTGGTAAGGGGCA (nucleotides 1488 to 1502) primers.

For N-terminal constructions, the Q and P domain fragments were converted to contain an *Xba*I site 5′ of the ACCA *Bbs*I-associated sequence to generate the sequence TCTAGACCA and a *Sty*I site downstream of the 3′ ACCA *Bbs*I-associated sequence to generate the sequence ACCACCTAGG by subcloning into the N-terminal adaptor vector pUC*oct*-2Nad. These Q and P domain sequences were subsequently excised by *Xba*I and *Sty*I double digestion and inserted into the *Xba*I site of pCG*oct*-2ΔN^{6}. For C-terminal constructions, the ACCA/TGGT Q domain and P domain *Bbs*I fragments were cloned into the C-terminal adaptor vector pUC*oct*-2Cad following the modified Oct-2 sequence cgcatcaacCCA CCA*TA*GTCTTCGGATCC, which contains C-terminal Oct-2 POU domain coding sequences (lowercase letters) fused to an ACCA *Bbs*I cleavage site (boldface), a *Bbs*I recognition site (underlined), a translational termination codon (italics), and a *Bam*HI cleavage site (GGATCC). The C-terminal Q or P domain sequences were then excised by *Sal*I (in the POU domain coding sequences) and *Bam*HI double digestion and recombined with an appropriate pCG*oct*-2 derivative. The N-terminal Q domain sequence in pCG*oct*-2ΔN^{4} and C-terminal P domain sequences in pCG*oct*-2ΔC^{8} were prepared by these N-terminal and C-terminal *Bbs*I strategies, respectively.

Different combinations of N-terminal and C-terminal se-

quences were generated by recombining appropriate constructs at the unique *Sfi*I site within the sequences encoding the central POU domain. In these constructs, the N-terminal and the C-terminal truncations were derived from ΔN^6 and ΔC^2, respectively.

To construct the GAL4(1–94) DNA-binding domain expression vector, the region encoding GAL4 positions 1 to 94 (4) was amplified by PCR with 5′ CACCCTAG[GATGAAGC TACTGTCTTC] and 3′ GCTCTAGAGCCTGGCAG[TGT TAACAATGCT] primers. (The primer sequences homologous to the GAL4 nucleotide sequence are bracketed.) The amplified product was cleaved at the *Sty*I and *Xba*I recognition sites (underlined) and cloned into the pCG vector at the unique *Xba*I site, regenerating the downstream *Xba*I site for in-frame fusions to heterologous coding sequences. In the final pCG-GAL4(1–94) vector, an in-frame termination codon and *Bbs*I recognition site with an ACCA cleavage site (all underlined) were introduced immediately downstream of the *Xba*I site in the sequence TCTAGACCATAGTCTTCGGATCC with a 3′ *Bam*HI site. With this vector, the amino acid sequence LPGSRP is generated between the GAL4(1–94)-encoding and heterologous sequences. To prepare pCG-GAL4 (1–94)1×Q, pCG-GAL4(1–94)2×Q, pCG-GAL4(1–94)1×P, and pCG-GAL4(1–94)2×P, one or two tandem *Bbs*I Q domain- or P domain-encoding fragments, as indicated, were inserted into the ACCA/TGGT *Bbs*I cleavage site in pCG-GAL4(1–94). In all cases, the nucleotide sequence of fragments derived from PCR was verified.

Reporter constructs. The β-globin reporter construct $p\beta\Delta^{36}6\times oct^{+}sph^{-}$ ($p\beta\Delta^{36}6\times$B20dpm2/−52) contains the human β-globin promoter truncated immediately upstream of the TATA box and six copies of the SV40 B-element octamer motif 52 bp upstream of the site of transcriptional initiation (29). The c-*fos* reporter pc-*fos*/−56/4×G17M contains four synthetic GAL4-binding sites (G17M [33]) upstream of the TATA box of the mouse c-*fos* promoter. This plasmid was derived from c-*fos*-CATΔ−56 (p301 −56), which contains mouse c-*fos* promoter sequences (−56 to +109 relative to the transcriptional start site) fused to the chloramphenicol acetyltransferase (CAT) gene (3). A fragment containing four G17M GAL4-binding sites, with a single A residue at the junction between binding sites, was prepared by self-ligation of the 18-nucleotide complementary oligonucleotides CGACGGAG TACTGTCCTC and GTCGGAGGACAGTACTCC and inserted into the unique *Sma*I site of pUC119 after filling in the resulting 4-nucleotide 5′ overhangs. The sequence containing four copies of the G17M GAL4-binding site was then excised with *Kpn*I and *Bam*HI, flush ended, and inserted between flush-ended *Hin*dIII and *Sal*I sites in c-*fos*-CATΔ−56. The internal reference plasmid pα4×(A+C) has been described previously (29).

Transient-expression assay. HeLa cells were transfected by the calcium phosphate coprecipitation method as previously described (32), with two minor modifications: the cells were seeded at twice the former density, and the transfected cells were harvested earlier (at 32 to 36 h posttransfection) than previously described. The β-globin (0.5 μg), internal reference pα4×(A+C) (4 ng), pCG effector (0.75 μg), and pUC119 carrier DNA (up to 10 μg of total DNA) plasmids were transfected into 7.5×10^5 cells on 60-mm dishes. To adjust expression of the different Oct effector proteins to comparable levels, some of the pCG effector plasmids were diluted up to 50-fold with a solution containing the same concentration of the empty expression vector pCG. A sequential threefold titration of effector plasmid concentration was included in analyses of some activators.

To assay the activity of GAL4 DNA-binding domain-containing activators, the pc-*fos*/−56/4×G17M reporter plasmid (0.5 μg) was used instead of a β-globin reporter, because transcription from our β-globin reporter constructs was activated by the GAL4 activators without added GAL4-binding sites, probably through one or more cryptic GAL4-binding sites in the vector sequences.

The transfected cells were harvested, and both RNA and protein extracts were prepared as described previously (32). RNA expression was measured by RNase protection as described previously (32). The c-*fos* probe is protected by correctly initiated transcripts over 128 nucleotides (12). A mixture of α-globin (α132) and β-globin (β350) probes was used to analyze the β-globin gene reporter transfection. A mixture of α-globin (α98) and c-*fos* (*fos*128) probes was used to analyze the c-*fos*-CAT gene reporter transfection. Quantitation of the results was performed with a Fuji BAS2000 bio-image analyzer.

Effector protein expression and QA complex analysis. The quality and quantity of the expressed effector proteins were monitored by an electrophoretic mobility shift assay essentially as described previously (32). The binding reactions were performed by first incubating the cell extract in 10 mM Tris (pH 8.0)–60 mM NaCl–1 mM dithiothreitol–1 mM EDTA–0.04% Nonidet P-40–8% glycerol–2.5% fetal bovine serum–0.3 μg of poly(dI-dC) per μl for 15 min on ice. The probe (10,000 cpm) was then added, and the reaction mix was incubated for a further 20 min. The samples were fractionated on a 5% polyacrylamide gel containing 0.25× Tris-borate buffer. For the reaction mixes containing the GAL4-derived proteins, 5 mM $MgCl_2$ and 20 μM $ZnSO_4$ were also included. Threefold serial dilutions of the sample displaying the highest DNA-binding activity were included in the same electrophoretic mobility shift assay to obtain a dose-response curve for binding activity. The radioactivities of the DNA-protein complexes, which were quantified with a Fuji BAS1000 and BAS2000 bio-image analyzer, were converted to relative DNA-binding activities by extrapolating them to the standard dose-response curve. The amounts of the complexes generated by truncated forms of the various effector proteins were never more than 15% of that of the full-sized complexes, and these complexes were included in the quantitations. The complexes that migrate more slowly than the major monomer DNA-protein complexes (the QA complexes; see below) were assumed to contain a single Oct protein per complex.

The radiolabeled probes for electrophoretic mobility shift analyses were made by PCR with a 5′-end-labeled oligonucleotide primer, followed by polyacrylamide gel electrophoresis purification. The Oct-2-binding site probe was a 1×B20dpm2 site from the SV40 enhancer (1). The GAL4-binding site probe was a single G17M 17-mer site.

RESULTS

Localization of the Oct-2 activation domains. We and others have previously shown that multiple tandem copies of the SV40 enhancer octamer motif do not activate the β-globin mRNA promoter in HeLa cells, which express Oct-1 but not Oct-2 (9, 28, 29). Ectopic expression of Oct-2, but not Oct-1, leads to efficient stimulation of transcription, and this stimulation is dependent on both N- and C-terminal Oct-2 sequences (29). To localize the Oct-2 N- and C-terminal activation domains more precisely, we analyzed the activation potentials of a series of Oct-2 truncations in a transient-transfection assay. In this assay, HeLa cells were transfected with (i) a β-globin reporter, $p\beta\Delta^{36}6\times oct^{+}sph^{-}$ (29), which

contains six copies of the SV40 enhancer B-element octamer motif positioned upstream of the β-globin TATA box, (ii) a pCG *oct*-2 cDNA expression plasmid based on the cytomegalovirus promoter, and (iii) an α-globin internal reference plasmid, called pα4×(A+C) (29). After transient expression, the transfected cells were divided into two portions. From one portion, we prepared RNA to measure transcriptional activation, and from the other, we prepared protein extracts to measure the quality and quantity of the expressed effector protein (32). RNA samples were analyzed by an RNase protection assay to monitor the levels of correctly initiated transcripts from the β-globin reporter and the α-globin internal reference. Protein extracts were subjected to an electrophoretic mobility shift assay to monitor Oct protein expression. This assay revealed that the majority (more than 85%) of the protein-DNA complexes displayed mobilities consistent with expression of full-length effector proteins.

Figure 1 shows the results of an RNase protection assay (Fig. 1A) and the structures of the truncated Oct-2 effectors along with the quantitation of both the activation and effector protein expression assays (Fig. 1B). The precise structures of the Oct-2 truncations are described in Table 1. A threefold serial titration of the Oct-2 effector plasmid indicated that, under our assay conditions, both the levels of β-globin gene transcription and the amount of effector DNA-binding activity increased nearly proportionally with increasing amounts of the transfected effector plasmid (Fig. 1A and B, lanes 2 to 4). At the highest effector concentration analyzed, Oct-2 activated transcription more than 60-fold. The Oct-2 titration allows us to compare the transcriptional activity of each of the various mutant proteins with that of the parental Oct-2 protein expressed at a similar level (see Fig. 1B). The N-terminal fusion of a short peptide encoding an epitope from the influenza virus hemagglutinin protein (29), which is present in some of the truncations, did not show any significant effect on Oct-2 activity (Fig. 1A, lane 5). In contrast to Oct-2, the Oct-2 DNA-binding domain alone was inactive in this assay (Oct-2$\Delta N^6\Delta C^2$; Fig. 1A, lane 19).

Lanes 6 to 10 in Fig. 1A show the activities of a series of truncations in the N-terminal region. Truncation of the N-terminal 94 amino acids (ΔN^1, lane 6) did not impair activation by Oct-2, as previously described (29). An additional 34-amino-acid deletion between a 66-amino-acid region rich in glutamine residues (26%) and the POU domain did not impair activation by Oct-2 either (ΔN^3 and ΔN^4; Fig. 1A, lanes 7 and 8). (The ΔN^3 and ΔN^4 truncations are identical except for an additional proline residue immediately upstream of the 66-amino-acid segment in the ΔN^4 protein; see Table 1.) However, a further N-terminal truncation of 17 amino acids, extending into the 66-amino-acid glutamine-rich region, had a significant deleterious effect on activation of the β-globin promoter (ΔN^5; Fig. 1A, lane 9), although this truncation is less deleterious than truncation of the entire N-terminal domain (Fig. 1A, compare lane 9 with lane 10, ΔN^6). Thus, the minimal N-terminal domain resides within the 66-amino-acid-long glutamine-rich region between amino acids 95 and 160 inclusive. The amino acid sequence of this glutamine-rich activation domain, which we refer to as the Q domain, is shown in Fig. 2.

We mapped the minimal C-terminal activation domain by assaying the ΔC series of truncations (Fig. 1A and B, lanes 11 to 18). Internal truncations between the C terminus of the POU domain (amino acid 358) and amino acids 380 (ΔC^3), 404 (ΔC^4), and 426 (ΔC^5) did not impair activation by Oct-2 when normalized to the level of effector protein expression (Fig. 1, lanes 11 to 13). But when the internal C-terminal truncation in

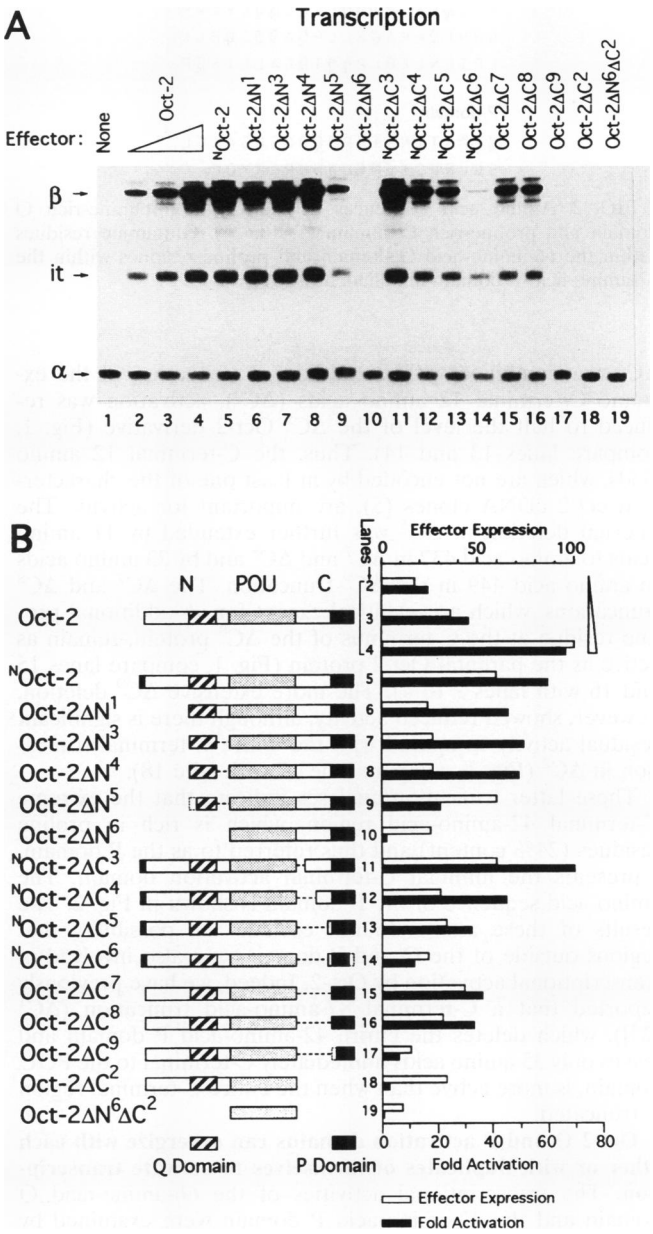

FIG. 1. Oct-2 transcriptional activation domains reside within a 66-amino-acid glutamine-rich domain and 42-amino-acid proline-rich domain. (A) Transient-transfection assay of transcriptional activation by Oct-2 and its truncation derivatives. The β-globin reporter plasmid p$\beta\Delta^{36}$6×oct$^+$sph$^-$ was transfected into HeLa cells together with the internal reference plasmid pα4×(A+C) and pCG effector plasmid, and the resulting RNAs were analyzed by RNase protection as described in Materials and Methods. Oct-2 activation was analyzed at three different amounts of effector plasmid. Bands corresponding to correctly initiated reference α-globin transcripts (α) and reporter β-globin transcripts (β) and incorrect β-globin vector transcripts (it) are indicated. (B) Structures of the truncated proteins (left) along with quantitation of their transcriptional activities and expression levels (right). The quantitation of effector expression relative to the highest level of Oct-2 expression as measured by DNA-binding activity (open bars) and of the fold transcriptional activation relative to the basal expression level in the absence of exogenous effector protein (solid bars) were determined as described in Materials and Methods.

Q domain

TGSQLAGDIQQLLQLQQLVLVP
GHHLQPPAQFLLPQAQQSQPGL
LPTPNLFQLPQQTQGALLTSQP

P domain

PPPATTNSTNPSPQQGSHSAIGL
SGLNPSTGPGLWWNPAPYQP

FIG. 2. Amino acid sequences of the Oct-2 glutamine-rich Q domain and proline-rich P domain (5, 18, 24). Glutamine residues within the 66-amino-acid Q domain and proline residues within the 42-amino-acid P domain are shaded.

ΔC^5 was combined with an additional truncation of the extreme C-terminal 12 amino acids (ΔC^6), activation was reduced to half the level of the ΔC^5 Oct-2 derivative (Fig. 1, compare lanes 13 and 14). Thus, the C-terminal 12 amino acids, which are not encoded by at least one of the characterized *oct*-2 cDNA clones (5), are important for activity. The internal deletion in ΔC^5 was further extended by 11 amino acids to amino acid 437 in ΔC^7 and ΔC^8 and by 23 amino acids to amino acid 449 in the ΔC^9 truncation. The ΔC^7 and ΔC^8 truncations, which are identical except for an additional proline residue at the C terminus of the ΔC^8 protein, remain as active as the parental Oct-2 protein (Fig. 1, compare lanes 15 and 16 with lanes 2 to 4). The more extensive ΔC^9 deletion, however, showed reduced activity, although there is significant residual activity compared with the entire C-terminal truncation in ΔC^2 (Fig. 1, compare lane 17 with lane 18).

These latter truncation analyses indicate that the extreme C-terminal 42-amino-acid region, which is rich in proline residues (24% content) and thus referred to as the P domain, represents the minimal C-terminal activation domain. The amino acid sequence of the P domain is shown in Fig. 2. The results of these assays do not exclude the possibility that regions outside of the Q and P domains are also involved in transcriptional activation by Oct-2. Indeed, we have previously reported that a C-terminal 87-amino-acid truncation (ΔC^1 [29]), which deletes the entire 42-amino-acid P domain and leaves only 35 amino acids immediately C-terminal to the POU domain, is more active than when the entire C-terminal region is truncated.

Oct-2 Q and P activation domains can synergize with each other or with duplicates of themselves to activate transcription. The transcriptional activities of the 66-amino-acid Q domain and the 42-amino-acid P domain were examined by combining them with the DNA-binding POU domain in different positions and in different combinations. Figure 3 shows the results of a transcription assay (Fig. 3A) and the structures of the activators along with quantitation of the levels of activation and effector protein expression (Fig. 3B; see also Table 1).

The Q.POU.P activator (Oct-2$\Delta N^4\Delta C^8$) contains the minimal 66-amino-acid Q domain and the 42-amino-acid P domain N and C terminal of the DNA-binding POU domain, respectively, as illustrated in Fig. 3B. The Q.POU.P protein activated the β-globin promoter as effectively as the parental Oct-2 protein (compare lanes 4 and 5 with lanes 2 and 3 in Fig. 3A and B). Truncation of either the Q or P domain of the Q.POU.P protein led to a dramatic reduction in activity (Fig. 3, lanes 6 and 7). The Q and P domains therefore synergize with each other to activate transcription efficiently, as described previously for the parental Oct-2 N- and C-terminal regions (29).

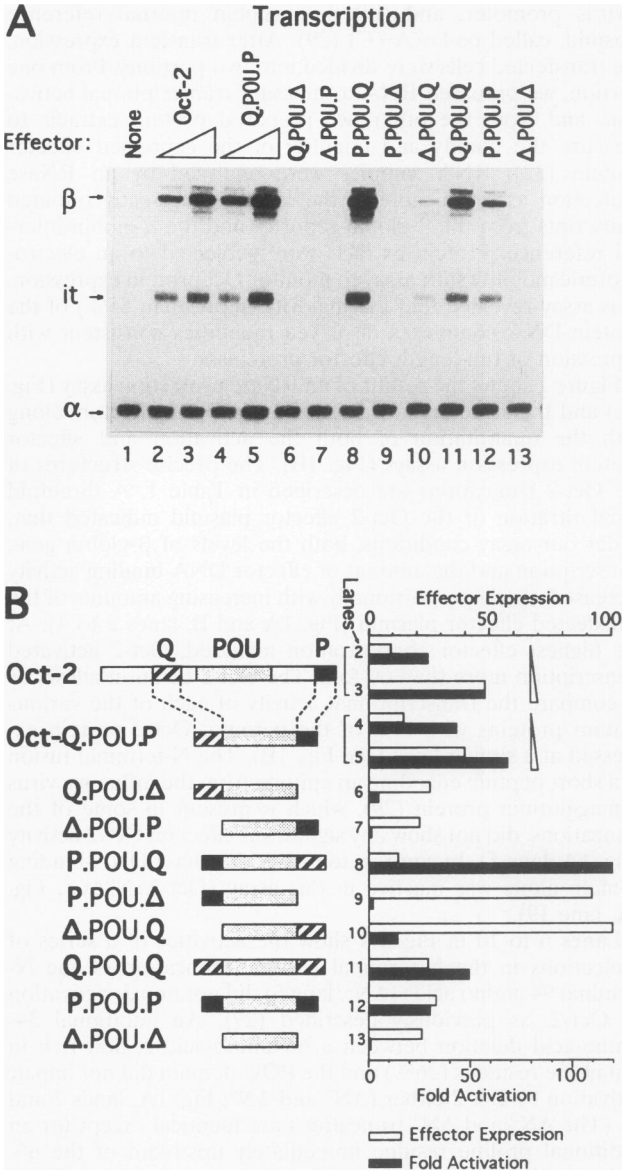

FIG. 3. Oct-2 Q and P domains synergize with each other or duplicates of themselves to activate transcription. (A) Transient-transfection assay of transcriptional activation by proteins containing the 66-amino-acid Q domain and/or the 42-amino-acid P domain. HeLa cells were transfected with the β-globin reporter and the α-globin internal reference plasmids along with pCG effectors as indicated, and the resulting RNAs were analyzed by RNase protection as described in the Fig. 1 legend. Oct-2 and Oct-Q.POU.P effector plasmids were transfected at two different amounts. Bands corresponding to correctly initiated α-globin transcripts (α) and β-globin transcripts (β) and incorrect β-globin vector transcripts (it) are indicated. (B) Effector structures (left) and quantitation of their transcriptional activity and levels of expression (right). The quantitation of effector protein expression relative to that of Δ.POU.Q as measured by DNA-binding activities (open bars) and of fold transcriptional activation relative to the basal level without exogenous effector proteins (solid bars) is shown.

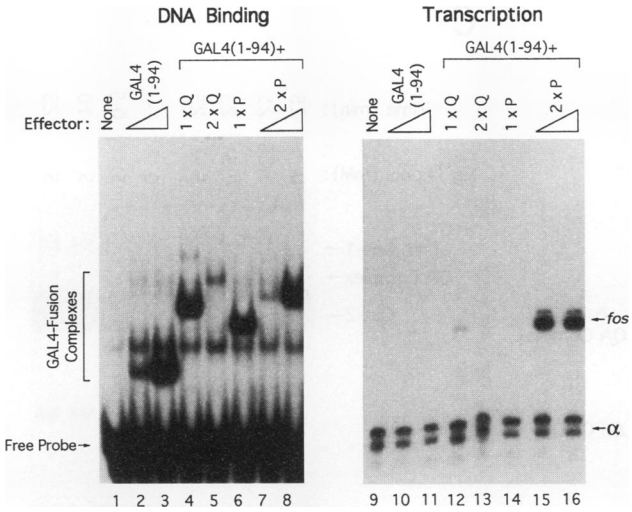

FIG. 4. The 42-amino-acid P domain can activate transcription in conjunction with the heterologous GAL4 DNA-binding domain. The results of a transient-expression assay of transcriptional activation by the GAL4 fusion proteins are shown. The c-*fos* reporter pc-*fos*/−56/4×G17M plasmid was transfected into HeLa cells together with the internal reference plasmid pα4×(A+C) and pCG effector plasmids as indicated (see Materials and Methods). Lanes 1 to 8, assay of transiently expressed proteins. Protein extracts were prepared from a portion of the transfected cells and subjected to an electrophoretic mobility shift assay as described in Materials and Methods. Lanes 9 to 16, assay of transcriptional activation. Cytoplasmic RNA isolated from transfected cells was analyzed by RNase protection. Bands corresponding to correctly initiated α-globin transcripts (α) and c-*fos* transcripts (*fos*) are indicated.

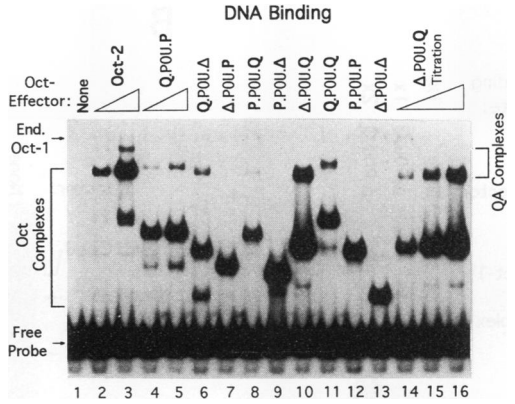

FIG. 5. Oct-2 Q domain directs formation of distinct more slowly migrating QA (Q activation domain-associated) complexes in an electrophoretic mobility shift assay. Activators containing the Q and/or P domain were analyzed by an electrophoretic mobility shift assay as described in Materials and Methods. Protein extracts were made from a portion of the transfected cells that were analyzed for RNA expression (Fig. 3). Lanes 14 to 16, in vitro threefold titration of the Oct-Δ.POU.Q protein. From the dose-response curve that was obtained from this titration, the relative DNA-binding activity of each effector protein, which is indicated in Fig. 3B by open bars, was determined. The positions of the major protein-DNA complexes which contain individual Oct activators (Oct complexes) and the more slowly migrating QA complexes are identified by brackets. End., endogenous.

To investigate the importance of the position of these activation domains for their synergistic action, the Q and P domains in Q.POU.P were exchanged to generate P.POU.Q. The P.POU.Q protein activates transcription more efficiently than the parental Oct-2 or Q.POU.P proteins and again relies on both the P and Q domains for optimal activity (Fig. 3, lanes 8 to 10). Duplications of the Q and P activation domains were also tested by generating Q.POU.Q and P.POU.P proteins. Both of these proteins were far more active than the corresponding N- or C-terminally truncated proteins containing a single activation domain (Fig. 3, compare lane 11 with lanes 6 and 10 and lane 12 with lanes 7 and 9). Thus, the Q and P domains can function in at least two locations with respect to the DNA-binding domain and can synergize not only with each other but also with duplicates of themselves to activate transcription. The positions of the Q and P domains can, however, affect the transcriptional activity of the proteins (e.g., compare Q.POU.P [lane 4] with P.POU.Q [lane 8] at similar effector expression levels). Nonetheless, these results indicate that the Q and P domains are functional modules that can be assembled together with the DNA-binding POU domain to constitute a functional activator without strict constraints.

The 42-amino-acid P domain functions with the heterologous GAL4 DNA-binding domain. We tested whether the Oct-2 Q and P domains, which synergize to generate a potent Oct-2 activator, can activate transcription independently of the POU domain by individually fusing one or two tandem copies of the 66-amino-acid Q domain and 42-amino-acid P domain sequence to the heterologous GAL4 DNA-binding domain (residues 1 to 94) (4). The activity of these GAL4 fusion proteins was assayed on a c-*fos* reporter construct containing four tandem GAL4-binding sites.

The results of this assay are depicted in Figure 4. Lanes 1 to 8 of Fig. 4 show an electrophoretic mobility shift assay showing that the GAL4 DNA-binding domain-containing effectors were properly expressed. Lanes 9 to 16 of Fig. 4 show the results of transcriptional activation. As expected, the GAL4 DNA-binding domain alone showed little or no transcriptional activity (Fig. 4, compare lanes 10 and 11 with lane 9). Fusion of a single Q or P domain to the GAL4 DNA-binding domain resulted in a low level of activity (Fig. 4, compare lane 9 with lanes 12 and 14). Transcription was activated 40-fold, however, when two P domains were fused in tandem to the GAL4 DNA-binding domain (Fig. 4, compare lanes 15 and 16 with lane 14). Thus, the P domain represents an autonomous activation domain that can activate transcription effectively when fused to a heterologous DNA-binding domain but only when duplicated. Curiously, two Q domains fused to the GAL4 DNA-binding domain produced low levels of activation (Fig. 4, lane 13) even though the protein was expressed at a higher level than the lower level of the GAL4(1–94)2×P protein assayed in lane 15 (Fig. 4, compare lanes 5 and 7), where the GAL4(1–94)2×P protein is already very active. These results are consistent with the ability of DNA-binding domains to influence the activity of activation domains.

Activators containing an active glutamine-rich activation domain generate distinct more slowly migrating complexes in an electrophoretic mobility shift assay. An unexpected result of the electrophoretic mobility shift assays used to measure the quantity and quality of effector protein expression was the appearance of a distinct set of complexes, called QA complexes (for Q activation domain-associated complexes), that migrate more slowly than the major protein-DNA complexes with Q domain-containing activators. An example of such an electrophoretic mobility shift assay is shown in Fig. 5, which represents the assay for the activation assay shown in Fig. 3. An example of the appearance of the QA complex can be seen in

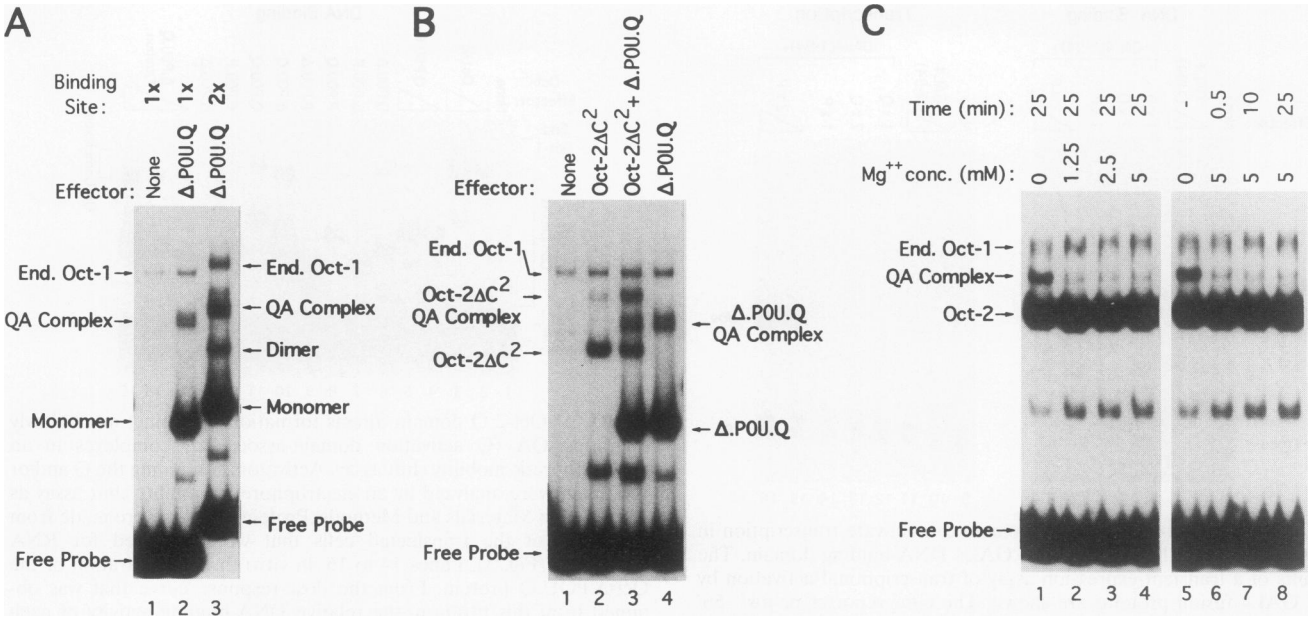

FIG. 6. Characterization of the more slowly migrating QA complexes by electrophoretic mobility shift assay. (A) Electrophoretic mobility shift assay of the Oct-Δ.POU.Q activator on probes containing one (1×) or two (2×) octamer-binding sites. Protein extracts were made from transfected cells and subjected to an electrophoretic mobility shift assay as described in Materials and Methods. The positions of the endogenous Oct-1 complex (End. Oct-1), the QA complex, the complexes containing one (Monomer) or two (Dimer) molecules of the Oct-Δ.POU.Q protein, and free probe are indicated to the left for the probe containing one octamer-binding site and to the right for the probe containing two octamer-binding sites. (B) Positions of the QA complexes generated by coexpressed Q domain-containing activators of different sizes. The Oct-2ΔC^2 and Oct-Δ.POU.Q proteins were expressed separately (lanes 2 and 4, respectively) or together (lane 3). The positions of the major Oct-2ΔC^2 complex and its QA complex are shown on the left, and those of the major Δ.POU.Q complex and its QA complex are shown on the right. (C) Effect of addition of magnesium chloride on the formation of the QA complex. To the Oct-2-containing samples shown in lanes 1 to 4, magnesium chloride was added for the entire 25-min binding reaction period at 0 to 5 mM concentration, as indicated above the lanes. For the Oct-2-containing samples shown in lanes 5 to 8, the binding reaction mixes lacked magnesium chloride (lane 5) or were made 5 mM magnesium chloride immediately (lane 6) or 10 or 25 min (lanes 7 and 8, respectively) before the electrophoresis.

lanes 4 and 5 of Fig. 5 with the Q.POU.P activator. In this instance, the QA complex migrates very close to where the Oct-2 complex migrates (Fig. 5, compare lanes 3 and 4). The mobility of the QA complex varies in correspondence to the mobility of the associated Oct-2-related protein. For example, compare the mobilities of the QA complexes and the Q.POU.P and Q.POU.Δ complexes in lanes 5 and 6 of Fig. 5. The QA complexes also appear only with proteins containing the glutamine-rich Q domain (Fig. 5, lanes 2 to 6, 8, 10, and 11) and not with the other proteins (lanes 7, 9, 12, and 13). The QA complexes also appear with the Q domain-containing Oct-2 truncations described in Fig. 1 but not with those proteins lacking the Q domain (data not shown). These more slowly migrating complexes suggest that a protein interacts specifically with the glutamine-rich Q domain in the electrophoretic mobility shift assay.

Further characterization of the QA complex is shown in Fig. 6. One possible mechanism by which the QA complex might be generated is the association of two Q domain-containing activators, as has been shown previously with the Sp1 Q-rich activation domains (20). To test such a possibility, the mobility of the QA complex was compared with that of a dimer protein-DNA complex that was formed on a probe containing two tandem octamer-binding sites (Fig. 6A). On both probes, however, the QA complex (Fig. 6A, lanes 2 and 3) migrates considerably more slowly than the dimer complex (lane 3). Thus, in contrast to the self-multimerization activity of the Sp1 Q-rich activation domains, the Oct-2 Q domain does not apparently generate the QA complex by directing association with another Oct-2-related protein.

To test this possibility further, however, we analyzed the results of coexpressing two differently sized Q domain-containing activators, Oct-2ΔC^2 and Δ.POU.Q, as shown in Fig. 6B. We expected that if the QA complexes result from association of the two Oct-2-related activators, then coexpression of two Q domain activators of different sizes would generate a unique QA complex of intermediate mobility because of the formation of heterodimers. The Oct-2ΔC^2 and Δ.POU.Q proteins did indeed form QA complexes of different mobilities when expressed separately (Fig. 6B, lanes 2 and 4). When coexpressed, however, they did not generate an additional unique QA complex of intermediate mobility (Fig. 6B, lane 3). Thus, the QA complex apparently contains a single activator protein and is instead generated through association with a heterologous cellular factor or by Q domain-dependent modification of the activator protein.

During analysis of the QA complexes, we noted an inhibitory effect of magnesium (Mg^{2+}) on QA complex formation, as shown in Fig. 6C for the Oct-2 QA complex. The presence of 1.25 to 5 mM Mg^{2+} during the 25-min incubation period led to inhibition of QA complex formation (Fig. 6C, lanes 1 to 4). This inhibitory effect could be obtained even when Mg^{2+} was added approximately 0.5 min before loading of the polyacrylamide gel and electrophoresis (Fig. 6C, compare lanes 6 and 8). This rapid inhibition of QA complex formation by Mg^{2+} suggests that if the QA complex is generated by modification of

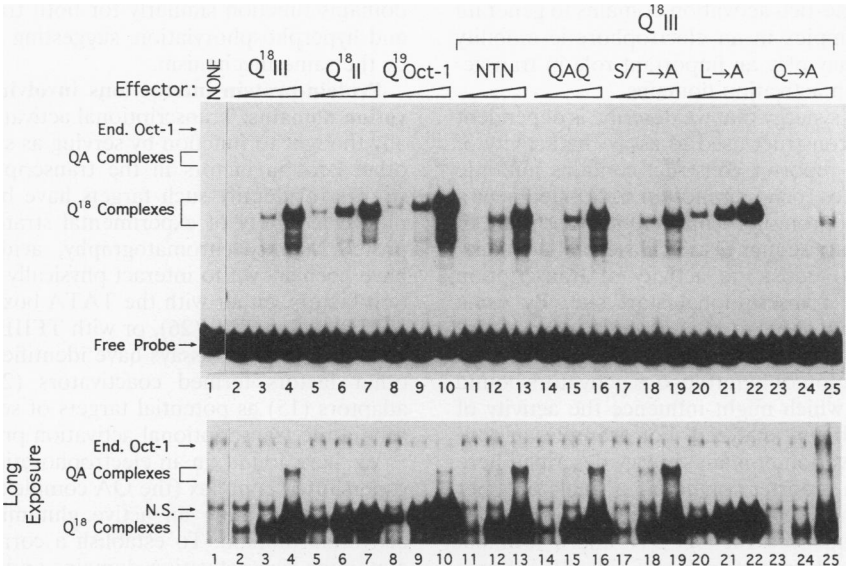

FIG. 7. QA complex formation by activators containing the Q^{18}III, Q^{18}II, and Q^{19}Oct-1 segments and the Q^{18}III(NTN), Q^{18}III(QAQ), Q^{18}III(S/T→A), Q^{18}III(L→A), and Q^{18}III(Q→A) mutant Q^{18}III segments. A threefold serial titration of effector plasmids was transfected into HeLa cells, and protein extracts were prepared. The positions of the major monomer (Q^{18} complexes), QA (QA complexes), endogenous Oct-1 (End. Oct-1), and nonspecific (NS) complexes are indicated. The bottom panel shows a part of a longer exposure of the same gel shown in the top panel.

the protein, it must be very rapidly reversed, a model we do not favor. It is more likely that the QA complex is generated by an interaction of the Q domain with a heterologous cellular factor that is sensitive to magnesium ions.

The aforementioned studies show that formation of the QA complex is dependent on the Oct-2 Q domain. To establish further a correlation between transcriptionally active glutamine-rich activation domains and formation of the QA complex, we compared the ability of eight synthetic glutamine-rich activation domains, four transcriptionally active and the others weak or inactive, to form QA complexes. We show in the accompanying article (30) that an 18-amino-acid glutamine-rich segment of the Oct-2 Q domain (NLFQLPQQTQ GALLTSQP; called Q^{18}III) can strongly activate transcription when tandemly repeated and fused to the Oct-2 POU DNA-binding domain. However, a neighboring 18-amino-acid glutamine-rich segment (Q^{18}II) or a 19-amino-acid glutamine-rich segment (Q^{19}Oct-1) derived from Oct-1 and highly similar to Q^{18}III failed to activate transcription effectively. The activity of the Q^{18}III segment is not severely affected when the two out of five glutamine residues in the QTQ sequence [Q^{18}III(NTN)] are converted to asparagine or when one [Q^{18}III(QAQ)] or all three [Q^{18}III(S/T→A)] threonine and serine residues present in this segment are converted to alanine residues. In contrast, activity is lost when the four leucine residues [Q^{18}III(L→A)] or all five glutamine residues [Q^{18}III(Q→A)] are converted to alanine residues (30). Thus, although all but the Q^{18}III(Q→A) synthetic activation domain contain a similar density of glutamine residues, they display very different transcriptional activities.

Figure 7 shows short and long exposures of an electrophoretic mobility shift assay of HeLa cell extracts containing transiently expressed proteins with four tandem copies of the Q^{18}III segment or its derivatives both N and C terminal of the POU domain. The Q^{18}III proteins (Fig. 7, lanes 2 to 4) and transcriptionally active mutant Q^{18}III(NTN) (lanes 11 to 13), Q^{18}III(QAQ) (lanes 14 to 16), and Q^{18}III(S/T→A) (lanes 17 to 19) proteins formed a QA complex, whereas the weak or inactive Q^{18}II (lanes 5 to 7), Q^{19}Oct-1 (lanes 8 to 10), and Q^{18}III(L→A) (lanes 20 to 22) proteins failed to form a QA complex as efficiently as Q^{18}III (lanes 2 to 4). The ability of the Q^{18}III(NTN) protein to form the QA complex efficiently shows that formation of the QA complex is not strictly correlated with the density of glutamine residues. The Q^{18}III(Q→A) protein, however, which shows little transcriptional activity (30) and contains no glutamines in the Q^{18}III segment, gave an ambiguous result. This protein consistently generates a smeary background in the electrophoretic mobility shift assay which intensifies both a QA-like complex and the most slowly migrating endogenous Oct-1 complex common to all the lanes (compare the endogenous Oct-1 band in lane 25 with that in lanes 1 to 24 of Fig. 7). Thus, it is difficult to compare the ability of the Q^{18}III(Q→A) protein to form a QA complex with that of the other Q^{18}-related proteins.

Together, these results suggest that the formation of a QA complex is reflective of a process, such as interaction with a target protein in the transcriptional machinery, that is important for glutamine-rich activation domains to activate transcription. The ability of the Q^{18}III(Q→A) protein to form an apparently weak QA complex suggests that glutamine residues are not absolutely essential for QA complex formation and that formation of a QA complex may be necessary but not sufficient for efficient transcriptional activation by these activators.

DISCUSSION

We have delineated and characterized two separate activation domains in Oct-2, an N-terminal glutamine-rich Q domain and a C-terminal proline-rich P domain. In our assay, they can synergize with each other or with duplicates of themselves to activate transcription independently of their precise position with respect to the DNA-binding domain. During the course of these experiments, we identified a factor that interacts specif-

ically with active glutamine-rich activation domains to generate the supershifted QA complex in an electrophoretic mobility shift assay. This factor may play an important role in transactivation by glutamine-rich activation domains.

The activation domain synergy that we describe is dependent on the precise reporter construct used to assay the activity of the Oct-2 proteins. Our reporter construct contains multiple tandem Oct-2-binding sites (octamer motifs) positioned near a minimal β-globin mRNA promoter truncated just upstream of the TATA box; no auxiliary sequences, such as distal enhancer elements, are present to boost the activity of transcription factors bound near the transcriptional start site. By using multiple tandem binding sites in the absence of a distal enhancer, the activity of transcription factors can be augmented without introduction of heterologous activators bound to a complex enhancer, which might influence the activity of the transcription factor being analyzed. For example, in contrast to the Q and P activation domain synergy described here and elsewhere (29), on a reporter containing a single octamer motif positioned near the β-globin TATA box together with distal SV40 enhancer sequences, the Oct-2 N- and C-terminal regions are functionally redundant in HeLa cells (19). Some assays in this latter study also did not detect the activity of the C-terminal P domain, even though, as shown here, the P domain is sufficient to activate transcription in conjunction with either the Oct-2 POU or GAL4 DNA-binding domain. It thus appears that other transcription factors bound to the promoter or enhancer can have a profound effect on how the individual Oct-2 activation domains contribute to the overall transcriptional activity of Oct-2. Such differences are probably important in generating diversity in the response of different promoters to individual activator proteins.

Modularity of transcriptional activator structure. Previous studies have revealed that transcriptional activators contain modular units for DNA binding and transcriptional activation, which can be exchanged among heterologous activators (21). Furthermore, reiteration of an activation domain can also lead to a significant increase in activity (8, 14, 25, 27). The Oct-2 Q and P domains demonstrate the modular and flexible nature of multiple distinct activation domains within a single activator. To constitute an activator, the Q and P domains can be mixed or matched together with the DNA-binding POU domain as if they were independent building blocks. We imagine that such flexible and modular structural features were important in the course of evolution for generating flexibility in the assembly of differing activation domains and DNA-binding domains into functional transcriptional activators.

The Oct-2 Q and P domains may be related to the glutamine-rich activation domains of Sp1 (6) and the proline-rich activation domain of CTF/NF-I (17), respectively. Indeed, the Oct-2 and Sp1 glutamine-rich activation domains both show approximately 25% glutamine content and are also rich in leucines plus serines or threonines. Consistent with this similarity, when substituted for the entire Oct-2 N-terminal region, the Sp1 glutamine-rich domain B (6) synergizes with the Oct-2 C-terminal region to activate transcription (31). We have previously shown that transcriptionally active, but not inactive, Oct-2 molecules are hyperphosphorylated in vivo (29). This correlation has been further extended to the active Q.POU.P protein, which is hyperphosphorylated, and its inactive deletion derivative Δ.POU.P and Q.POU.Δ proteins, which are not hyperphosphorylated (31). Consistent with a functional similarity between the Oct-2 and Sp1 glutamine-rich activation domains, the Sp1 glutamine-rich activation domain also induces hyperphosphorylation in concert with the C-terminal Oct-2 sequences (31). Thus, the Oct-2 and Sp1 glutamine-rich domains function similarly for both transcriptional activation and hyperphosphorylation, suggesting that they may function by the same mechanism.

Protein-protein interactions involving glutamine-rich activation domains. Transcriptional activation domains are generally thought to function by serving as surfaces to interact with other protein factors in the transcription process (21), and studies to identify such targets have been intensive and employed a variety of experimental strategies. For example, by protein affinity chromatography, acidic activation domains have been shown to interact physically with general transcription factors, either with the TATA box-binding component of TFIID, called TBP (26), or with TFIIB (16). Furthermore, in vitro transcription assays have identified either TFIIB (16) or other factors termed coactivators (22), mediators (2), or adaptors (15) as potential targets of sequence-specific activators in the transcriptional activation process.

We have found, in an electrophoretic mobility shift assay, a supershifted complex (the QA complex) that is specific to Oct proteins containing an active glutamine-rich transcriptional activation domain. To establish a correlation between active glutamine-rich activation domains and the appearance of the supershifted QA complex in the electrophoretic mobility shift assays, we made use of a series of well-defined active and inactive multimerized activation domains (30). Of the eight cases tested, in seven cases the appearance of the QA complex supershift clearly correlated with the presence of a functional activation domain, even though most of these activation domains differ by only a few point mutations within the reiterated 18- or 19-amino-acid segment (see accompanying article [30]); in the eighth case, Q^{18}III(Q→A), the result was ambiguous for technical reasons (see Fig. 7). This Oct-2-associated QA complex is likely to be generated through interaction of the functional glutamine-rich activation domains with another cellular factor. This factor represents another candidate target of a transcriptional activation domain.

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