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Differential Transcriptional Activation by Oct-1 and Oct-2: Interdependent Activation Domains Induce Oct-2 Phosphorylation

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Summary

The ubiquitous Oct-1 and lymphoid Oct-2 POU homeodomain transcription factors bind to the same DNA sequence but differ in their activation potential. Oct-2 is a positive, negative, or neutral regulator of β-globin transcription depending on the position and sequence of multimerized binding sites. To activate transcription, Oct-2 relies on two interdependent nonacidic domains, an N-terminal glutamine-rich region and a C-terminal serine-, threonine-, and proline-rich region. Oct-1 also contains a functional glutamine-rich region but fails to activate β-globin transcription in our assay because the Oct-1 C-terminus is inactive, indicating that differential activation by Oct-1 and Oct-2 is determined by the combination of multiple activation domains. Oct-2 displays a unique phosphorylation pattern that is absent from molecules lacking one or the other activation domain, suggesting the activation domains have a role in inducing protein phosphorylation.

Introduction

How do transcription factors that bind to the same *cis*-acting element differentially activate transcription? Studies of yeast transcription factors have shown that DNA binding and transcriptional activation domains can be separated and interchanged between transcriptional regulators (Brent and Ptashne, 1985; reviewed by Ptashne, 1988). Some activation domains, e.g., those of the yeast *trans*-activators GAL4 and GCN4, are characterized by a high concentration of acidic amino acids (Hope and Struhl, 1986; Ma and Ptashne, 1987; Hope et al., 1988), and can activate transcription of various promoters in cells of diverse eukaryotic species (reviewed by Ptashne, 1988). In these cases, the specificity of transcriptional activation is conferred by the DNA binding domain, which tethers the activation domain to the promoter. Hence these prototypic transcriptional activators cannot explain differential activation of transcription by factors that bind to the same DNA sequence. In this latter case the specificity of activation must reside in the activation domain.

The two octamer motif (ATGCAAAT) binding proteins Oct-1 (also referred to as OTF-1, OBP100, and NFIII) and Oct-2 (OTF-2) are a model system to address this question. Generally, when the octamer motif is placed in the context of an RNA polymerase II mRNA-encoding promoter, such as the immunoglobulin or β-globin gene promoters, it displays a lymphoid-specific activity that correlates with the expression pattern of Oct-2 (see Tanaka et al., 1988; Schaffner, 1989, for references). When placed in the context of an RNA polymerase II small nuclear RNA (snRNA) promoter, however, the octamer motif is active in nonlymphoid cells, thus correlating with the expression pattern of the ubiquitous Oct-1 protein (Mattaj et al., 1985; Mangin et al., 1986; Ares et al., 1987; Tanaka et al., 1988). Because the octamer motif is unusual in its ability to activate the U2 snRNA promoter—neither a GAL4 *trans*-activator nor three SV40 enhancer elements tested could stimulate a U2 snRNA promoter—Tanaka et al. (1988) proposed that Oct-1 and Oct-2 have intrinsically different activation potentials: Oct-2, like GAL4, readily activates mRNA promoters, and Oct-1 can enhance snRNA transcription.

Oct-1 (Sturm et al., 1988) and Oct-2 (Ko et al., 1988; Clerc et al., 1988; Müller et al., 1988; Scheidereit et al., 1988) are POU homeodomain proteins related to the pituitary transcription factor Pit-1/GHF-1 (Ingraham et al., 1988; Bodner et al., 1988) and the nematode developmental gene product *unc-86* (Finney et al., 1988). The POU proteins are characterized by an extended region of similarity containing a C-terminal homeodomain and an N-terminal POU-specific region (Herr et al., 1988). This entire region, referred to as the POU domain, is 87% identical between Oct-1 and Oct-2 and is responsible for their DNA binding activities (Sturm et al., 1988; Clerc et al., 1988; Ko et al., 1988; Sturm and Herr, 1988). To either side of the centrally located POU domain the similarity between Oct-1 and Oct-2 is less extensive, although the N-terminal regions are both glutamine rich, as are activation domains in the transcription factor Sp1 (Courey and Tjian, 1988), and the C-terminal regions contain high concentrations of serines, threonines, and prolines.

Müller et al. (1988) demonstrated that Oct-2 expressed in nonlymphoid cells (HeLa) can activate a β-globin promoter containing an octamer motif, thus providing an assay to compare the transcriptional activation potentials of Oct-1 and Oct-2. By overexpression of Oct-1 and Oct-2 in HeLa cells, we show here that Oct-2 possesses an intrinsic ability, lacking in Oct-1, to activate a β-globin promoter containing multimerized octamer motifs. Activation by Oct-2 is dependent on two separate regions that lie N- and C-terminal of the Oct-2 POU domain. The Oct-1 N-terminus can functionally replace the Oct-2 N-terminus, but Oct-1 itself fails to activate β-globin transcription because the C-terminus is inactive. Thus, here the specificity of transcriptional activation by Oct-1 and Oct-2 is determined by the combination of multiple functional domains. Analysis of the proteins expressed from various Oct-2 wild-type and mutant constructs and Oct-2–Oct-1 chimeric constructs reveals specific protein phosphorylation that correlates with the ability to stimulate mRNA transcription.

Results

Oct-2 but Not Oct-1 Activation of a β-Globin Promoter in HeLa Cells

Oct-1 and Oct-2 activation of truncated β-globin promoters containing multimerized octamer motifs was examined by

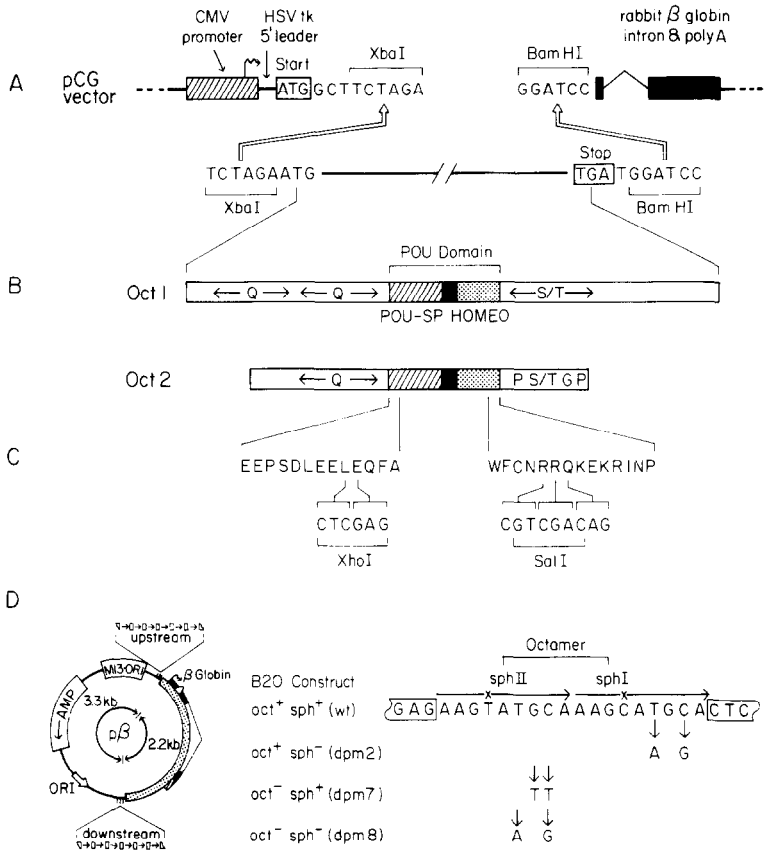

Figure 1. Effector and Reporter Gene Constructs

(A) The first line shows the structure of the pCG expression vector with, from left to right, the human CMV promoter, HSV tk gene 5′ untranslated leader and initiation codon, the unique XbaI (engineered downstream of the initiation codon) and BamHI sites, and the rabbit β-globin segment containing splicing and poly(A) addition signals. Below is shown the sequence of the engineered XbaI and BamHI sites upstream and downstream of the Oct coding sequences.

(B) A schematic diagram of Oct-1 and Oct-2 protein structures. The DNA binding domains (POU domain) are shown with the POU-specific box (hatched box), linker region (filled box), and POU-homeo box (stippled box). The sequences flanking the POU domain are indicated by open boxes. The N-terminal regions of Oct-1 and Oct-2 that are rich in glutamines (Q) are indicated. A portion of the Oct-1 C-terminal region is rich in serines and threonines (S/T), whereas the 111 amino acid long C-terminal portion of Oct-2 can be crudely divided into four segments: the first 32 amino acids are 20% proline (labeled "P"), the next 22 amino acids contain a potential leucine zipper motif (Landschulz et al., 1988; Clerc et al., 1988; Müller et al., 1988; Scheidereit et al., 1988) and are 55% serine or threonine (S/T), the third segment (16 amino acids) contains a stretch of 8 glycines (G), and the final 41 amino acids are both proline (25%) and serine/threonine (55%) rich (P).

(C) The amino acid sequences at the N-terminal and C-terminal end of the POU domains are shown. The indicated amino acid sequences are identical in Oct-1 and Oct-2. The XhoI and SalI sites were engineered as shown below by the nucleotide sequences.

(D) The structure of the β-globin reporter plasmids pβΔ^{127} (previously called pβe^{-}; Ondek et al., 1987) and pβΔ^{36} is shown to the left. These plasmids contain the human β-globin gene (stippled box) inserted into the polylinker (hatch marks) of pUC119. Synthetic enhancers composed of six wild-type or mutant copies of the SV40 enhancer B element (the 6×B20 series) containing overlapping octamer and sph motifs (shown to the right) are located either upstream or 2.2 kb downstream of the β-globin transcriptional start site depicted by a wavy arrow (see Tanaka et al., 1988 and Experimental Procedures). The sequence of the wild-type B20 repeat is shown to the right. When the B20 repeat is reiterated, the boxed nucleotides form XhoI restriction sites and extend the identity with the SV40 enhancer sequence by two nonessential nucleotides to 20 bp. The sph and octamer motifs are indicated by arrows and a bracket, respectively. The changes in the double point mutants dpm2, dpm7, and dpm8 are shown below the wild-type sequence.

transient expression of *oct*-1 and *oct*-2 cDNAs in HeLa cells. We cloned the *oct*-1 (Sturm et al., 1988) and *oct*-2 (Clerc et al., 1988) cDNAs in the expression vector pCG as illustrated in Figure 1 and described in Experimental Procedures. The *oct* expression plasmids were transfected into HeLa cells together with the series of β-globin reporter plasmids shown in Figure 1D and an α-globin internal reference plasmid. Correctly initiated transcripts were quantitated by RNAase protection analysis. Each of the reporter plasmids contains a β-globin promoter truncated to immediately upstream of the TATA box (Δ^{36}; Baumruker et al., 1988) or upstream of the CCAAT and [illegible] for Oct-1 or Oct-2 activation were six tandem copies of wild-type or mutant SV40 B elements (Tanaka et al., 1988) inserted either upstream or 2.2 kb downstream of the truncated β-globin promoters (Figure 1D).

The 18 bp SV40 enhancer B element (Figure 1D) contains two overlapping sets of motifs: the tandemly repeated 9 bp sph motifs I and II, which are both required for enhancer activity in HeLa cells (Ondek et al., 1988; Fromental et al., 1988), and the octamer motif formed by the junction of the two sph motifs, which is sufficient for B cell–specific enhancer activity (Fromental et al., 1988; Tanaka et al., 1988). A series of double point mutations (dpm) allows the activities of the sph and octamer motifs to be separated (Tanaka et al., 1988). The dpm2 mutation (called oct^{+}sph^{-} here) only inactivates the sph motifs and therefore functions in B cells but not in HeLa cells. This construct was used to test transcriptional activation by Oct-1 and Oct-2. The dpm7 mutation (oct^{-}sph^{+}) inactivates the octamer motif but not the sph motifs and therefore does not affect activity in HeLa cells. The dpm8 mutation (oct^{-}sph^{-}) affects both sets of motifs and is inactive in both HeLa cells and B cells.

The results of a cotransfection experiment using the pβΔ^{36} reporter plasmids are shown in Figure 2A. The level of β-globin transcripts from the oct^{+}sph^{-} construct,

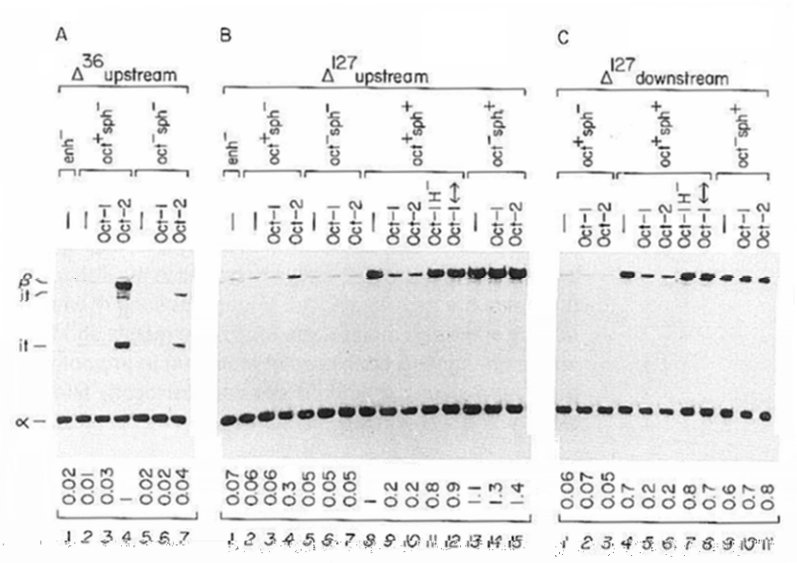

Figure 2. Activation and Repression of the β-Globin Promoter by Cloned Oct-1 and Oct-2 Proteins

β-globin reporter plasmids were transfected into HeLa cells together with an internal reference pα 4×(A+C) and with or without a pCG construct expressing either Oct-1 or Oct-2 polypeptides. Cytoplasmic RNA was analyzed by RNAase protection as described in Experimental Procedures. Bands corresponding to correctly initiated α-globin transcripts (α), β-globin transcripts (β), and transcripts derived from the β-globin reporter plasmids but initiated within the upstream vector sequence (it) are indicated. In (A), (B), and (C) are shown transfections with derivatives of the pβΔ^{36} (A), pβΔ^{127} upstream (B), and pβΔ^{127} downstream (C) reporter constructs. The binding sites present in each reporter construct are indicated above the lanes (refer to Figure 1); enh⁻, no inserted binding sites. The effector plasmid used is indicated by the first row above the panels: –, no effector plasmid; Oct-1, pCG*oct*-1; Oct-2, pCG*oct*-2; Oct-1H⁻, pCG*oct*-1H⁻ (a homeobox DNA binding defective point mutant); Oct-1 ↔, pCG*oct*-1 ↔ (an inverted *oct*-1 coding sequence construct). The relative levels of correctly initiated β-globin transcription are shown above the lane numbers. Quantitation was done by averaging the results of two independent transfection experiments in (A) and one experiment in (B) and (C) and are normalized to the oct⁺sph⁻ construct cotransfected with the Oct-2 effector plasmid (lane 4) in (A) and to the pβΔ^{127} upstream 6× oct⁺sph⁺ construct without cotransfected effector plasmid ([B], lane 8) in (B) and (C). Note that the specific acitivity of the β350 probe used in (A) was identical to that of the α132 probe but, in (B) and (C), the specific activity of the β350 probe was 10-fold lower than that of the α132 probe.

in the absence of a cotransfected *oct* expression vector, is similar to the enhancerless control, confirming that endogenous HeLa cell Oct-1 does not activate this β-globin promoter construct (Figure 2A, lanes 1 and 2). Upon cotransfection with pCG*oct*-1, the level of β-globin transcripts remains relatively unchanged (lane 3), but cotransfection of pCG*oct*-2 results in a 50- to 100-fold increase in β-globin transcripts (lane 4). The Oct-2 activation is dependent on the octamer motif because with the mutant octamer construct oct⁻sph⁻ (lanes 5–7), correctly initiated β-globin transcripts disappear (compare lanes 4 and 7). Oct-2 does, however, continue to stimulate expression of incorrectly initiated transcripts that produce aberrantly spliced products (labeled "it"). This stimulation may be due to degenerate octamer motifs elsewhere in the plasmid that activate nearby cryptic promoters.

Figures 2B and 2C show the activity of Oct-1 and Oct-2 when the octamer motifs are placed either upstream or at a distance downstream of the pβΔ^{127} construct. Activation by Oct-2 but not Oct-1 is observed when the octamer binding sites are placed upstream of the β-globin promoter (Figure 2B, lanes 1–4), and this activity is octamer motif dependent (lanes 5–7). In contrast, as indicated previously by Müller et al. (1988), when the binding sites are placed at a distance from the promoter, Oct-2 does not activate transcription (Figure 2C, lane 3). The distance dependence of Oct-2 activation is consistent with the B cell–specific activity of the SV40 octamer motif (presumably mediated by Oct-2) because this octamer motif is over 10 times more active when placed in the upstream position as opposed to the distal position (Tanaka et al., 1988).

Competitive Binding of Oct-1 and Oct-2 Molecules to the Octamer Motif Represses the Activity of Overlapping sph Motifs

Using the SV40 B element with either active or inactive sph motifs overlapping the octamer motif (see Figure 1D), we have examined the effect of sequence context on the transcriptional activity of Oct-1 and Oct-2. The experiments shown in Figures 2A and 2B (lanes 1–4) demonstrate that when the sph motifs are inactive (oct⁺sph⁻), Oct-2 is an activator and Oct-1 has little or no effect. When the sph motifs are active, however, as in the oct⁺sph⁺ construct, cotransfection with the Oct-1 or Oct-2 expression plasmids reduces β-globin expression 3-to 5-fold either when the oct⁺sph⁺ sites are placed upstream (Figure 2B, compare lane 8 with lanes 9 and 10) or downstream (Figure 2C, compare lane 4 with lanes 5 and 6) of the Δ^{127} β-globin promoter. The reduction in β-globin expression is relieved either by a mutation in the Oct-1 homeodomain (Oct-1H⁻) that prevents binding to the octamer motif (Sturm and Herr, 1988) or inversion of the Oct-1 coding sequences in the expression vector (Oct-1 ↔). The reduction in β-globin expression is also relieved in *cis* by the oct⁻sph⁺ mutation that inactivates the octamer motif but not the sph motifs (see Figure 2B, lanes 13–15 and Figure 2C, lanes 9–11). These results are consistent with repression of sph motif activity by competitive binding of the Oct factors to the octamer motif, thus displacing or otherwise inactivating factors bound to the sph motifs. Consistent with this interpretation, immunoprecipitation of Oct-1 from transfected cells reveals that the transfected Oct-1 protein is at least 10-fold more abundant than endogenous Oct-1 (data not shown).

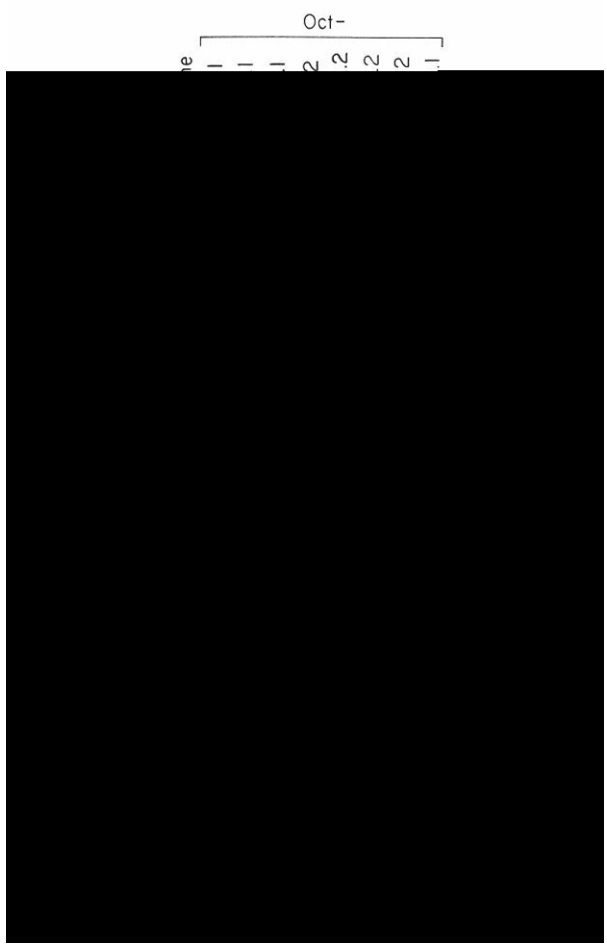

Figure 3. Transcriptional Activation by Oct-1 and Oct-2 Chimeric Proteins

The activation assay is with the pβΔ^{36} 6×B20 dpm2 (oct$^+$sph$^-$) β-globin reporter plasmid transfected together with the effector constructs indicated above the lanes and was assayed as described in the legend to Figure 2. The structures of the chimeric molecules are shown below (Oct-2 sequences are hatched) with the quantitations of the activation assay (Act.) and the control repression assay (Rep.). The repression assay was performed by transfecting the pβΔ^{127} 6×B20 wt (oct$^+$sph$^+$) downstream construct together with the various pCG expression constructs. The levels of β-globin transcripts were normalized to the wild-type pCG*oct*-2 construct (activation) or to the control (labeled "None") without any cotransfected effector construct (repression).

The "repression assay" described above allows two important points to be made. First, the inability of Oct-1 to activate transcription in Figure 2A is not due to low level expression or improper localization of the Oct-1 protein, because Oct-1 can bind to a reporter plasmid and effectively interfere with β-globin expression. Thus, the inactivity of Oct-1 is intrinsic to the Oct-1 molecule. For this report we used the sph motif repression assay with the sph motifs in a downstream position (Figure 2C) to assay effective expression of each of the Oct-1 or Oct-2 derivatives described below. The second point is that an activator such as Oct-2 can either activate, repress, or have no effect on transcription depending on the promoter context. Thus, Oct-2 activates transcription when the sph motifs are inactive and the octamer binding sites are proximal to the start site of transcription (e.g., Figure 2A, lane 4); Oct-2 has no effect when the mutant sph motif construct oct$^+$sph$^-$ is placed at a distance (Figure 2C, lanes 1 and 3); and Oct-2 represses transcription when the sph motifs are active (Figure 2B, lanes 8 and 10; Figure 2C, lanes 4 and 6). The unexpected repression by Oct-2 in the upstream position can be accounted for by a weaker activator displacing a stronger activator bound to the sph motifs (compare the activity of Oct-2 [Figure 2B, lane 4] with the activity of the sph motifs [lane 8]). These results show that subtle changes (a double point mutation) in promoter sequences flanking a binding site can drastically alter the activity of a transcription factor.

The C-Terminus is Responsible for the Different Activation Potential of Oct-1 and Oct-2

To map the region(s) responsible for the different activation patterns of Oct-1 and Oct-2 (Figure 2A), we generated a series of chimeric Oct-1–Oct-2 molecules and assayed their activation potential. To construct chimeric Oct-1–Oct-2 proteins, unique restriction sites were created by introducing silent mutations within sequences shared by Oct-1 and Oct-2 (see Figure 1C). These sites divide the Oct-1 and Oct-2 molecules into an N-terminal region, central DNA binding POU domain, and a C-terminal portion (Figure 1B). Chimeric molecules are designated by a three digit system that specifies the origin of the three separate regions. Thus, Oct-2.1.1 contains the N-terminal region of Oct-2 fused to the Oct-1 POU domain and C-terminal region. The chimeric expression constructs were cotransfected into HeLa cells either with the pβΔ^{36} oct$^+$sph$^-$ construct to test for activation or with the pβΔ^{127} oct$^+$sph$^+$ downstream construct to assay repression.

As shown in Figure 3, transfer of the Oct-2 N-terminus or POU domain to Oct-1 fails to activate Oct-1 (lanes 2–4), but transfer of the Oct-2 C-terminus does activate Oct-1 (lane 5). Transfer of Oct-1 sequences to Oct-2 (lanes 6–9) displays the reciprocal result: Oct-2 proteins containing the Oct-1 N-terminus or POU domain are active (lanes 7 and 8), but the C-terminal exchange dramatically decreases Oct-2 activity (lane 9). This result identifies the C-terminal regions of Oct-1 and Oct-2 as the determinants that distinguish the ability of these two proteins to activate the β-globin promoter.

Both the N- and C-Terminal Portions of Oct-2 Cooperate to Generate a Functional Transcriptional Activator

The regions required for transcriptional activation by the Oct factors were examined by constructing a series of plasmids expressing truncated or internally deleted derivatives of the octamer binding proteins. The results of activation and repression by these mutated Oct proteins are shown in Figure 4. Deletion of either the N-terminal or C-terminal Oct-1 sequences does not create an activator (lanes 3 and 4), but both repress sph motif activity to a comparable level. Therefore, these results do not reveal a negative regulatory domain that can repress the activity of a separate Oct-1 activation domain as does the hor-

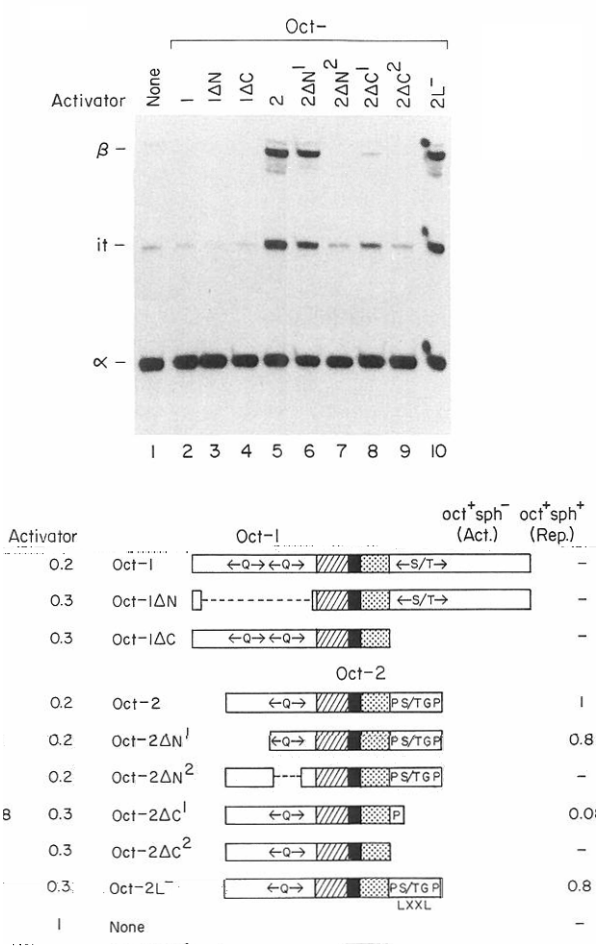

Figure 4. Transcriptional Activation of the β-Globin Promoter by Mutant Oct-1 and Oct-2 Proteins

The activation assay was performed by transfecting the β-globin reporter plasmid pβΔ^{36} 6×B20 dpm2 (oct$^+$sph$^-$) into HeLa cells together with the pCG expression construct indicated above the lanes and assaying expression as described in the legend to Figure 2. Below is shown schematically the structure of the Oct-1 and Oct-2 mutants and, to the right, quantitation of activation (Act.) and repression (Rep.) assays. In the repression assay, the pβΔ^{127} 6×B20 wt (oct$^+$sph$^+$) downstream construct was transfected into HeLa cells together with the mutant Oct-1 and Oct-2 expression construct as indicated. The levels of correctly initiated transcripts are normalized to levels produced upon cotransfection with the wild-type pCG*oct*-2 construct in the activation assay and to the control (labeled "None") without any expression construct cotransfected in the repression assay. "–" indicates less than 3% the level of Oct-2 activation. Quantifiable samples are the average of two experiments.

mone binding domain in steroid receptors such as the glucocorticoid receptor (Godowski et al., 1987; Hollenberg et al., 1987).

Although only the C-terminus distinguishes the activity of Oct-1 and Oct-2, activation by Oct-2 is dependent on both the N-terminal and C-terminal regions. Removal of 94 amino acids from the N-terminus of Oct-2 (Oct-2ΔN^1) has little effect on transcriptional activation (Figure 4, compare lanes 5 and 6), but internal deletion of the adjacent glutamine-rich (26% glutamine) 62 amino acids (Oct-2ΔN^2) dramatically inactivates Oct-2 (lane 7). A C-terminal 87 amino acid truncation (Oct-2ΔC^1) causes a 12-fold reduction in Oct-2 activity (lane 8), whereas further truncation of the remaining 35 amino acids to the C-terminus of the DNA binding domain (Oct-2ΔC^2) leaves no detectable activity (lane 9). All of these truncations showed comparable 3- to 5-fold repression activity, indicating no major effect of these truncations on expression and DNA binding. These results demonstrate that both the N-terminal glutamine-rich region and the C-terminal region, which contains patches of highly concentrated prolines, serines, and threonines (see legend to Figure 1), are required for optimal activation by Oct-2.

The 111 amino acid long C-terminal region of Oct-2 also contains a heptad repeat of 4 leucines that potentially form a "leucine zipper" (Landschulz et al., 1988; Clerc et al., 1988; Müller et al., 1988; Scheidereit et al., 1988). We tested the involvement of the leucine repeat for Oct-2 function by mutating the two internal leucine residues to isoleucine residues. Such a mutation debilitates C/EBP leucine zipper–mediated dimer formation (Landschulz et al., 1989). In Oct-2, however, this mutation has little effect on activity in HeLa cells (Figure 4, lane 10). Nevertheless, this putative leucine zipper structure could be important in other cell types such as B cells, where a protein that interacts with the Oct-2 leucine repeats may be expressed.

Oct-1 Contains a Functional Glutamine-Rich N-Terminus

The ability of Oct-1–Oct-2 chimeras carrying the N-terminus of Oct-1 to activate β-globin transcription (Figure 3) suggests that the glutamine-rich N-terminus of Oct-1 can participate in activation of this promoter. To verify this conclusion, we deleted the N-terminal Oct-1 sequences in the active Oct-1.1.2 and Oct-1.2.2 chimeras and tested their activity. As shown in Figure 5, deletion of the Oct-1 N-terminus does indeed inactivate these Oct chimeras (compare lanes 2 and 3, and 5 and 6). Furthermore, neither the Oct-1 nor Oct-2 POU domains activate the β-globin promoter in the pβΔ^{36} construct (lanes 4 and 7). Together, the experiments described in Figures 3–5 show that Oct-2 contains two regions that cooperate to activate transcription; Oct-1 contains a region functionally equivalent to the Oct-2 glutamine-rich N-terminal domain but differs from Oct-2 in the C-terminal region where it fails to display an equivalent activation potential.

Transcriptionally Active Oct Molecules Display Distinct Phosphorylation Patterns

The sph motif repression assay described above monitors the efficiency of activator expression, but it only tests for DNA binding activity. We therefore used an independent assay to monitor the quality of the expressed Oct proteins. For this second assay, we fused the N-terminus of 11 different Oct proteins to a small peptide epitope from the influenza virus hemagglutinin protein, for which a high affinity monoclonal antibody (12CA5) is available (Field et al., 1988; see Experimental Procedures). In addition to Oct-1 and Oct-2, we selected both active (Oct-1.1.2 and Oct-1.2.2) and inactive (Oct-2.1.1 and Oct-2.2.1) Oct-1–Oct-2 chimeras, several Oct-2 deletions (ΔN^2, ΔC^1, and

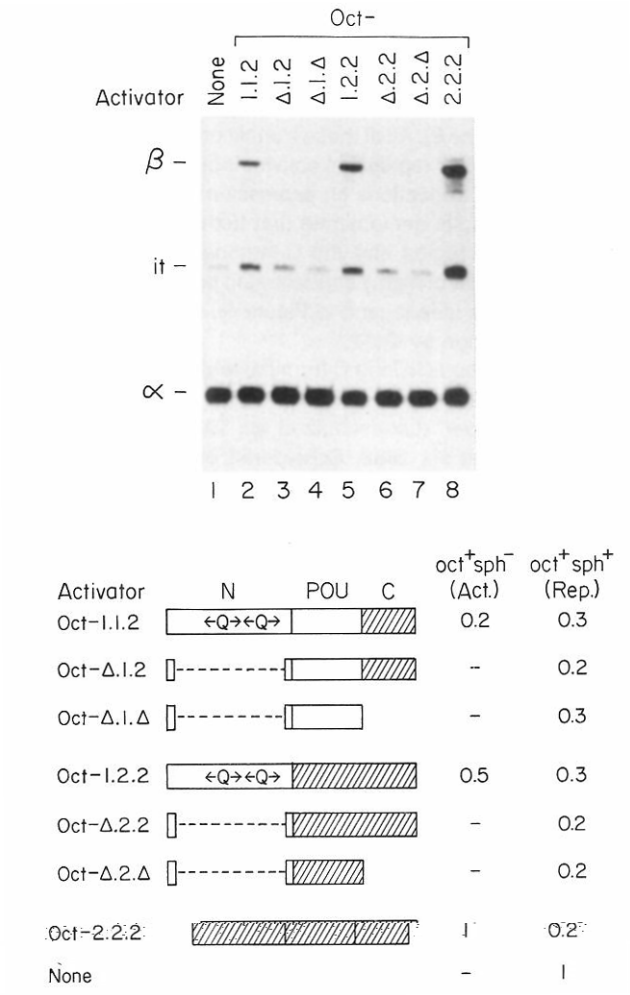

Figure 5. The Oct-1 N-Terminus Can Participate in Activation of the β-Globin Promoter

An activation assay with wild-type and deleted Oct-1 and Oct-2 chimeric molecules is shown. The pCG effector constructs were transfected as indicated above the lanes along with pβΔ[36] 6×B20 dpm2 (oct$^+$sph$^-$) and assayed as described in the legend to Figure 2. Below are shown the structures of Oct-1 and Oct-2 chimeric molecules and their deletion derivatives. The hatched regions are derived from Oct-2. The quantitation of the activation (Act.) and repression (Rep.) assays is shown to the right and was normalized as described in the legend to Figure 3.

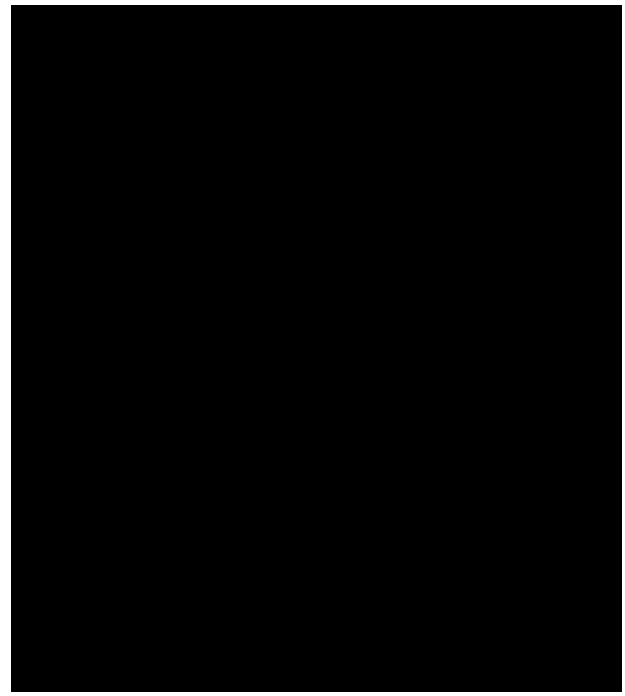

Figure 6. Immunoprecipitation of Recombinant Oct Proteins

HeLa cells (two 60 mm dishes for each sample) were transfected with the pCGN expression constructs indicated above the lanes together with pβΔ[36] 6×B20 dpm2 (oct$^+$sph$^-$), pα4×(A+C), and pUC119 (10 μg total per dish). One plate was analyzed for β-globin expression, and cells on the other plate were metabolically labeled with [^{35}S]methionine for 4 hr, lysed in RIPA buffer, and subjected to immunoprecipitation with the 12CA5 monoclonal antibody as described in the Experimental Procedures. The immunoprecipitates were analyzed by 8% SDS–polyacrylamide gel electrophoresis and fluorographed.

ΔC[2]), and the Oct-Δ.1.2 and Oct-Δ.2.2 chimeras lacking the Oct-1 glutamine-rich region. The assay for transcriptional activation by these epitope fusions revealed that their activity is unaffected by the presence of the short epitope (data not shown).

In parallel with the activation assay, duplicate transfections were labeled with ^{35}S, and the cell lysates were immunoprecipitated with the 12CA5 monoclonal antibody and fractionated by SDS–polyacrylamide gel electrophoresis. The results of this experiment are shown in Figure 6. Each polypeptide was expressed to a comparable level, although larger polypeptides were in general less well represented. Nevertheless, there is no obvious correlation between the levels of expression and transcriptional activity (e.g., the level of inactive Oct-2.2.1 [lane 7] is similar to the level of active Oct-1.1.2 [lane 8]). Furthermore, the construct with the lowest level of expression (Oct-1; lane 1) repressed sph motif activity as well as the other constructs (see Figures 2–5), suggesting that the lowest level of expression is already above saturation for occupancy of the octamer binding sites in the reporter constructs. Subcellular localization of these various polypeptides was examined by immunofluorescence with the 12CA5 antibody, and they were all localized in the nucleus (data not shown).

An unexpected result of the analysis shown in Figure 6 is the striking electrophoretic heterogeneity of the Oct-2 protein (lane 2) compared with the Oct-1 protein (lane 1). This heterogeneity is in the form of two sets of doublet bands and is also found in the active chimeric proteins Oct-1.1.2 and Oct-1.2.2 (lanes 8 and 10). In contrast, the inactive chimeras Oct-2.1.1 and Oct-2.2.1 (lanes 6 and 7) produce a single band similar to Oct-1 (lane 1). The deletion constructs show that if the N-terminal glutamine-rich region is deleted from the active proteins as in Oct-2ΔN[2] (lane 3), Oct-Δ.1.2 (lane 9), and Oct-Δ.2.2 (lane 11), the quantity of the electrophoretically retarded species is greatly reduced (about 20-fold); the C-terminal Oct-2 dele-

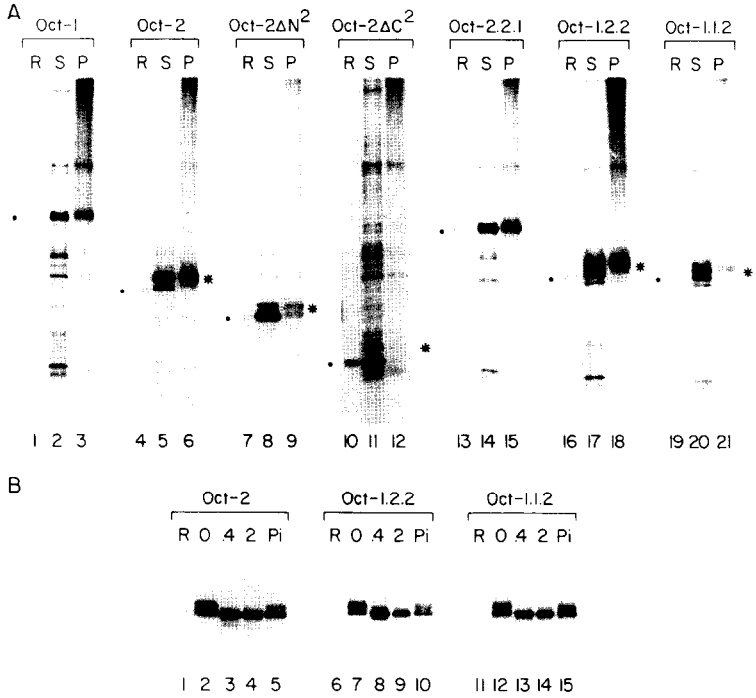

Figure 7. Phosphorylation of Recombinant Oct Proteins Expressed in HeLa Cells

(A) HeLa cells were transfected with the pCGN expression construct indicated above the lanes, metabolically labeled with either [^{35}S]methionine (lanes labeled "S") or [^{32}P]phosphoric acid (lanes labeled "P"), and the cells were lysed in RIPA buffer. Immunoprecipitates were prepared with the 12CA5 monoclonal antibody and analyzed on 8% SDS–polyacrylamide gels. For comparison, in vitro translated protein (lanes labeled "R") was prepared from the identical set of pCGN constructs as described in Experimental Procedures and subjected to immunoprecipitation. The dots indicate the positions of the immunoprecipitated in vitro translation products. The asterisks indicate the positions of major electrophoretically retarded species. The number of methionines per molecule vary from 9 residues in Oct-2ΔC^2, to 11 residues in Oct-2, Oct-2ΔN^2, Oct-1.1.2, and Oct-1.2.2, and 13 residues in Oct-1 and Oct-2.2.1. (B) Dephosphorylation of recombinant Oct proteins with potato acid phosphatase. The immunoprecipitates were prepared from ^{35}S-labeled HeLa cells transfected with the pCGN construct indicated above each panel. Immunoprecipitates were divided into four portions and subjected to potato acid phosphatase treatment. The samples contained immunoprecipitates incubated with buffer only (lanes 2, 7, and 12), 0.4 U (lanes 3, 8, and 13), or 2 U of potato acid phosphatase (lanes 4, 9, and 14), and 2 U of potato acid phosphatase in 0.2 M sodium phosphate (lanes 5, 10, and 15). Immunoprecipitates of in vitro translated proteins were included for comparison (lanes 1, 6, and 11). The positions of the panels have been adjusted to center the differently sized Oct proteins.

tions (lanes 4 and 5) also drastically reduce the level of the retarded species, but in both the N- and C-terminal deletions the retarded species do not entirely disappear. There is an apparent correlation, therefore, between the appearance of major electrophoretically retarded species and the ability to activate transcription in the β-globin HeLa cell assay. Consistent with this correlation, the retarded species is abundant in the active deletion Oct-2ΔN^1 (data not shown).

To characterize the retarded species further, we compared the mobility of in vivo and in vitro synthesized Oct proteins and labeled the proteins separately in vivo with ^{35}S and ^{32}P. As shown in Figure 7A, with each of seven Oct proteins analyzed, the in vitro translated protein (lanes R, identified by dots) generally comigrates with the faster migrating in vivo ^{35}S-labeled species (lanes S). Thus, the retarded species likely result from one or more modifications that do not occur during in vitro translation. The ^{32}P-labeled samples (lanes P) reveal that each of the Oct proteins is phosphorylated, but to varying extents. The total levels of ^{32}P labeling compared with ^{35}S labeling appear higher in Oct-1 (lanes 2 and 3), Oct-2 (lanes 5 and 6), and the Oct-1–Oct-2 chimeric proteins (compare lanes 14, 17, and 20 with 15, 18, and 21, respectively) compared with the inactive N- (lanes 8 and 9) and C-terminal truncations (lanes 11 and 12). These results indicate that the Oct molecules are efficiently phosphorylated if the N- and C-terminal regions deriving from either Oct-1 or Oct-2 are present. As in Figure 6, however, only the Oct-2 derivatives that can effectively activate the β-globin promoter display high levels of the electrophoretically retarded species, and these retarded species are considerably more phosphorylated than the species that comigrate with the in vitro translated proteins (compare lanes 5 and 6, lanes 17 and 18, and lanes 20 and 21; see asterisks). Thus, although Oct-1 and Oct-2 are both heavily phosphorylated, only Oct-2 and its active derivatives display the electrophoretic shift.

The correlation between the extent of Oct-2 phosphorylation and the electrophoretic mobility of the polypeptides implies that either the phosphorylation itself causes the slower migration or only the slower migrating forms are efficiently phosphorylated. To discriminate between these two possibilities, ^{35}S-labeled polypeptides were treated with potato acid phosphatase to remove O-linked phosphates. As shown in Figure 7B, the retarded Oct-2, Oct-1.2.2, and Oct-1.1.2 species (lanes 2, 7, and 12) all disappear upon treatment with phosphatase (lanes 3 and 4, 8 and 9, and 13 and 14) but not if the phosphatase inhibitor sodium phosphate is included during phosphatase treatment (lanes 5, 10, and 15). These results demonstrate that one or more O-linked phosphorylations are responsible for generating the Oct species with reduced electrophoretic mobility. High levels of the electrophoretically retarded species are dependent on the same Oct N-terminal and Oct-2 C-terminal regions that are involved in activation of transcription from the octamer motif containing the β-globin promoter. Thus, these phosphorylated Oct-2 species are probably those that efficiently activate the β-globin promoter.

Discussion

Comparison of the activities of the octamer motif in two different promoter contexts, the U2 snRNA promoter and the β-globin promoter, suggested that the activation domains of the octamer motif binding proteins Oct-1 and Oct-2 have evolved to stimulate transcription of different types of promoter complexes (Tanaka et al., 1988). The cloning of the genes encoding Oct-1 and Oct-2, along with the demonstration that expression of Oct-2 in HeLa cells can activate a cotransfected β-globin promoter (Müller et al., 1988), made it possible to test one prediction of this model: that Oct-2 contains a domain lacking in Oct-1 that specifies the ability to activate an mRNA promoter. The experiments described here are consistent with this prediction, as high level expression of Oct-2 but not Oct-1 can activate transcription from a β-globin promoter containing multimerized octamer motifs. The domain responsible for this difference lies within the C-terminus of Oct-2.

A caveat with this interpretation is that the N-terminus of Oct-1 (about 50 amino acids; see Experimental Procedures) is probably missing in the studies described here, and therefore it is possible that the N-terminus of Oct-1 performs an equivalent function to that of the C-terminus of Oct-2. This possibility seems unlikely, however. Assay of Oct-1 extended by a further 23 amino acids at the N-terminus also fails to activate the β-globin promoter (G. Das and M. T., unpublished data). Furthermore, the sequence similarities between these two evolutionarily related proteins and the interchangeability of the functionally important N-termini argue against a functional transposition of the C-terminus of Oct-2 to the N-terminus of Oct-1.

Oct-1 derived from the cloned *oct*-1 gene is functional in two in vitro assays: it forms a characteristic multiprotein–DNA complex with the herpes simplex virus (HSV) *trans*-activator VP16 (Vmw65) (Stern et al., 1989), and it activates adenovirus DNA replication (P. Verryzer and P. C. van der Vliet, personal communication). To date, however, it has not been possible to assay activation of the U2 snRNA promoter by Oct-1—an assay that would permit the reciprocal experiments to those described here to be performed. Accurate U2 snRNA transcription using cell extracts has not been reported, and assay of U2 snRNA promoter activation by cloned Oct-1 in vivo has been unsuccessful, probably owing to the presence of endogenous Oct-1. In vivo overexpression of Oct-1, Oct-2, or deletion mutants of these proteins (e.g., the DNA binding domains alone) neither stimulates nor represses U2 snRNA transcription in our assay (M. T., unpublished data). This result suggests that either the Oct-1 and Oct-2 DNA binding domains are sufficient to activate U2 snRNA transcription (hence no repression), and endogenous Oct-1 is already at saturating levels (hence no activation), or stable complexes are established on the transfected U2 promoter with endogenous Oct-1 before Oct factors from the cotransfected effector plasmids can be expressed. Consistent with the latter suggestion, U2 snRNA promoter complexes are very stable (Mattaj et al., 1985).

Differential Activation

The ability of Oct-2 to activate a lymphoid-specific promoter in HeLa cells (Müller et al., 1988; this study) shows that these nonlymphoid cells contain all the factors required for Oct-2 to activate transcription. Therefore, the differences in transcriptional activation by Oct-1 and Oct-2 in HeLa cells must rest upon different interactions with other transcription factors present in HeLa cells. The failure of high level expression of Oct-1 to activate the β-globin promoter argues that Oct-1 does not fail to activate a TATA box promoter in vivo simply because it is expressed at low levels, as was suggested because elevated levels of Oct-1 can activate transcription of an immunoglobulin promoter in vitro (LeBowitz et al., 1988). In vitro assays may not measure transcriptional activation potential as stringently as the in vivo assay, because the Oct-2 DNA binding domain, which is inactive in vivo (see Figure 5), is also active in vitro (M. T., unpublished data).

Previous results have shown that except for the presence or absence of the sph motifs neither the flanking sequences nor the precise position of the multimerized octamer motifs is critical for snRNA or β-globin activation (Tanaka et al., 1988; P. Reinagel and N. Hernandez, personal communication). Therefore the different activation potentials of Oct-1 and Oct-2 are unlikely to result from different interactions with factors bound to sequences adjacent to the octamer motif. Instead, the different activation potentials are probably the result of the intrinsic abilities of Oct-1 and Oct-2 to interact productively with the different classes of promoter complexes that form near or at the site of snRNA and mRNA transcription initiation (Tanaka et al., 1988). Consistent with this hypothesis, snRNA promoters differ considerably from mRNA promoters. They share a snRNA-specific proximal element (reviewed by Dahlberg and Lund, 1988), which is not stimulated by mRNA enhancer elements (Tanaka et al., 1988), and only RNA polymerase II transcription complexes that initiate on an snRNA promoter can later recognize the "3′ box" sequence that specifies the 3′ end of the transcripts (Hernandez and Weiner, 1986; Neuman de Vegvar et al., 1986; Hernandez and Lucito, 1988). Therefore, snRNA promoters are likely to have significantly different initiation complexes than mRNA promoters.

Although the activities discussed above are intrinsic to the Oct proteins and are not dependent on a strict sequence environment or position of the octamer motif, it is evident that these parameters can have important effects on the activities of the octamer motif binding proteins. For example, we have shown here that a double point mutation in the overlapping sph motifs or changes in the position of the octamer motifs can turn Oct-2 into a positive, negative, or neutral regulator of β-globin transcription (see Figure 2). Sequence-specific positive and negative effects by cloned transcription factors have also been observed with natural promoters and steroid receptors (Akerblom et al., 1988; Sakai et al., 1988), the bovine papilloma virus E2 protein (Stenlund and Botchan, 1990), and the Drosophila UBX homeodomain protein (Krasnow et al., 1989).

Subtle promoter context–dependent activities may also explain the ability of the octamer motif to activate the histone H2B promoter in response to the cell cycle (LaBella et al., 1988). Unlike in snRNA promoters, in the histone H2B promoter the octamer motif is positioned close to the

transcriptional start site immediately upstream of a canonical TATA box. This specific promoter context may allow the Oct-1 protein to activate a TATA box promoter. Indeed, recent results show that a β-globin reporter construct with the histone H2B octamer motif positioned immediately upstream of the TATA box (clone 5; Müller et al., 1988) is stimulated by overexpression of Oct-1, albeit to a lesser extent than by Oct-2 overexpression (M. T., unpublished data). This result shows that it was critical for the studies described here that promoter constructs that distinguish clearly between Oct-1 and Oct-2 were used.

Dissection of the abilities of Oct-1 and Oct-2 to activate the β-globin promoter reveals that it is the combination of multiple activation domains that results in the different potential of these two proteins to activate a β-globin promoter. Although the domains of Oct-1 required for activation of snRNA transcription are not known, the finding that Oct-1 and Oct-2 share interchangeable glutamine-rich activation motifs suggests that there may be common features to activation of an mRNA and snRNA transcription complex. Consistent with this suggestion, Sp1, another sequence-specific transcription factor implicated in U2 snRNA transcription (Ares et al., 1987; Janson et al., 1987), also contains glutamine-rich domains that activate mRNA transcription in cooperation with a C-terminal domain (domain D; Courey and Tjian, 1988).

The unique 111 amino acid long C-terminal Oct-2 activation domain is superficially unrelated to the acidic (Hope and Struhl, 1986; Ma and Ptashne, 1987; Hope et al., 1988; Triezenberg et al., 1988) or glutamine-rich (Courey and Tjian, 1988; this study) activating regions, as there are no acidic residues and only three glutamine residues. The major obvious quality of the Oct-2 C-terminus is that it contains separate patches of highly concentrated proline residues and serine/threonine residues and a region with a high concentration of both types of residues (see legend to Figure 1). In the *trans*-activator CTF/NF-I, the 100 amino acid long transcriptional activation domain is 25% proline but is not as serine/threonine-rich as the Oct-2 C-terminus (18% vs. 30%) (Mermod et al., 1989). Regions of high serine/threonine content are also observed in other homeodomain *trans*-activators (e.g., *bicoid* [Struhl et al., 1989]) and this structure may, therefore, reflect a new class of transcriptional activation domains. Nevertheless, the inactivity of the Oct-1 C-terminus, which contains a very serine/threonine-rich region (50% over 120 amino acids), suggests that either serine/threonine content alone is insufficient to create an activation domain, or the remainder of the Oct-1 C-terminus contains a negative regulatory domain.

Phosphorylation of Active Oct Proteins

Analysis of wild-type and mutant Oct proteins expressed in vivo reveals that the wild-type Oct-1 and Oct-2 proteins are phosphorylated, but only Oct-2 or Oct-1–Oct-2 chimeras that can activate the β-globin promoter display a shift in electrophoretic mobility upon phosphorylation (see Figure 7). The large reduction in Oct-2 phosphorylation upon deletion of either one or the other of the two interdependent activation domains (compare wild-type Oct-2 with Oct-2ΔN and Oct-2ΔC in Figure 7) suggests a role for these activation domains in Oct-2 phosphorylation. Where the Oct-2 phosphorylation sites are located, or whether it is the phosphorylated state that is competent to activate transcription, is not known. It is evident that because both Oct-1 and Oct-2 are phosphorylated, phosphorylation itself does not explain the different activities of these two proteins. The Oct-2–specific electrophoretic mobility shift that is induced by phosphorylation may, however, signal a change in conformation that permits activation of the β-globin promoter. It is intriguing that among Drosophila homeodomain proteins the activators *bicoid* and *fushi tarazu* also display a phosphorylation-induced electrophoretic shift (Driever and Nüsslein-Volhard, 1989; Krause et al., 1988), whereas the repressor *engrailed*, although phosphorylated, does not display such a change in electrophoretic mobility (Gay et al., 1988).

Phosphorylation has been implicated in transcriptional activation by other transcription factors. In some cases phosphorylation induces DNA binding activity, as with E2F (Bagchi et al., 1989), a cellular factor involved in adenovirus E2 promoter activation, and CREB, the cAMP response element binding protein (Yamamoto et al., 1988). In the case of the Oct factors it is unlikely that phosphorylation has a large effect on DNA binding, because the nonphosphorylated forms of the Oct factors bind DNA effectively (i.e., in vitro translated proteins; data not shown) and repress transcription in the in vivo sph motif assay. As with Oct-2, phosphorylation of the yeast heat shock transcription factor (HSF) upon heat shock correlates with transcriptional activation without apparently affecting DNA binding activity (Sorger and Pelham, 1988). Whether, like Oct-2, the activation domains of HSF are required for induction of phosphorylation is not known.

Analysis of transcriptional activation by Oct-1 and Oct-2 has revealed two indirect mechanisms by which proteins may activate transcription: protein–protein association and protein modification. In the first case, the presence of the HSV *trans*-activator VP16 confers upon Oct-1 the ability to activate the same octamer motif–containing β-globin promoters that are activated by Oct-2 (Tanaka et al., 1988). This reprogramming of Oct-1 results because VP16 forms a multiprotein–DNA complex with Oct-1 (O'Hare and Goding, 1988; Gerster and Roeder, 1988; Stern et al., 1989) and contains a GAL4-like acidic domain responsible for transcriptional activation of mRNA promoters (Triezenberg et al., 1988; Sadowski et al., 1988). In the second case, the studies described here suggest that Oct-2 becomes competent to activate transcription by protein phosphorylation, a modification that may either directly create an acidic activation domain or induce an allosteric change that creates or otherwise reveals an Oct-2 activating region. Because protein associations and protein modifications in most cases depend on the presence of a second protein (e.g., VP16 or a kinase), these types of activation pathways can contribute to temporal and spacial patterning of transcriptional activity without changes in the levels of the transcription factor itself.

Experimental Procedures

Effector Constructs

The expression vector pCG contains the human CMV promoter, the

HSV tk gene 5′ untranslated leader and initiation codon, rabbit β-globin gene splicing and polyadenylation signals, and the replication origin of SV40. This plasmid was constructed from the plasmid pSTC (Severne et al., 1988; kindly provided by Dr. S. Rusconi) by introducing the M13 phage replication origin from pBSM13$^+$ and then engineering a unique XbaI site downstream of the HSV tk gene initiation codon (see Figure 1A). To produce the pCG*oct*-1 and pCG*oct*-2 expression plasmids, the *oct*-1 and *oct*-2 cDNA coding sequences were inserted between the pCG XbaI and BamHI sites after engineering XbaI and BamHI sites upstream of the *oct* ATG codons (TCTAGAATG) and downstream of the TGA stop codons (TGATGGATCC), respectively. The *oct*-1 coding sequences begin at the first *oct*-1 AUG codon described previously (Sturm et al., 1988) and encode a 743 amino acid long Oct-1 protein. Analysis of 16 *oct*-1 cDNA clones (G. Das and W. Herr, unpublished data) has revealed that, although this initiation codon is probably used in an alternatively spliced form of the *oct*-1 mRNA, it is unlikely to encode full-length Oct-1; rather its estimated size on polyacrylamide gels suggests that it is lacking about 50 N-terminal amino acids. The longest *oct*-1 cDNA isolated reveals an upstream open reading frame extending for 42 amino acids with an AUG codon 23 amino acids upstream. The *oct*-2 coding sequences are derived from the plasmid pass5 (Clerc et al., 1988; R. Clerc and P. Sharp, personal communication); except for an extra 5 N-terminal amino acids, these sequences are identical to those of the Oct-2 protein expressed by Müller et al. (1988).

To prepare chimeric Oct proteins, XhoI and SalI sites, indicated in the plasmid names by (2) and (3), respectively, were introduced into the N-terminal and the C-terminal coding sequences of the Oct-1 and Oct-2 DNA binding domains (see Figure 1C). The deletion constructs were prepared as follows. The N-terminal deletion pCG*oct*-1ΔN is the ΔBH deletion described previously (Sturm et al., 1988) in the pCG*oct*-1(2) plasmid (meaning it has the XhoI site). The N-terminal truncation construct pCG*oct*-2ΔN^1 was constructed by removing the fragment between the unique XbaI site and the *oct*-2 HincII site (nucleotide 344; Clerc et al., 1988) from pCG*oct*-2(2). The plasmid pCG*oct*-2ΔN^2 was generated by removing the fragment between the NheI site (nucleotide 360) and SmaI site (nucleotide 545) from pCG*oct*-2(2). The C-terminal truncation constructs pCG*oct*-1ΔC, pCG*oct*-2ΔC^1, and pCG*oct*-2ΔC^2 were constructed by inserting an XbaI linker that contains stop codons in all three reading frames (CTAGTCTAGACTAG) into flush-ended DNAs digested at the PflMI site (nucleotide 1318) of pCG*oct*-1(2), HindIII site (nucleotide 1238) of pCG*oct*-2(3), or PstI site (nucleotide 1137) of

The chimeric constructs were generated by recombining *oct*-1, *oct*-2, and their deletion derivatives at the engineered XhoI and/or SalI sites (Figure 1C). Below are listed the designations of the chimeric proteins followed by the designations of the cDNA constructs, which also indicate the exact structure of each plasmid. Each plasmid is divided into the N-terminal, DNA binding (POU), and C-terminal portions, and the origin of each portion is indicated by "1" for Oct-1 and "2" for Oct-2. The presence of the engineered XhoI and SalI sites or their absence is indicated by (2), (3), and (0), respectively: Oct-2.1.1, pCG*oct*-2(2)1(0)1; Oct-1.2.1, pCG*oct*-1(2)2(3)1; Oct-1.1.2, pCG*oct*-1(0)1(3)2; Oct-1.2.2, pCG*oct*-1(2)2(0)2; Oct-2.1.2, pCG*oct*-2(2)1(3)2; Oct-2.2.1, pCG*oct*-2(0)2(3)1; Oct-Δ.1.2, pCG*oct*-1ΔN(2)1(3)2; Oct-Δ.2.2, pCG*oct*-1ΔN(2)2

396 and 403 (Clerc et al., 1988) were mutated to isoleucine codons (CTG to ATC and TTA to ATA, respectively) in the parent construct *oct*-2(2)2(3)2. The homeobox mutant pCG*oct*-1H$^-$ was generated by exchanging the HincII–NcoI fragment of pCG*oct*-1 with that of pBS*oct*-1(H-AAA) (Sturm and Herr, 1988).

The expression plasmid pCGN, which gives rise to an N-terminal translational fusion with a small epitope derived from the influenza virus hemagglutinin gene, was constructed by inserting double-stranded oligonucleotides encoding for the peptide SSYPYDVPDYASLGGPSR (Field et al., 1988) into the plasmid pCG at the unique XbaI site. The oligonucleotides were designed such that the insertion destroys the XbaI site downstream of the HSV tk initiation codon but maintains the XbaI site downstream of the peptide encoding sequence. The XbaI–BamHI fragments encoding the wild-type and mutant Oct proteins were introduced into the plasmid pCGN between the XbaI and the BamHI sites to generate the pCGN series of expression constructs for immunoprecipitation.

Reporter Constructs

The β-globin reporter plasmid containing the β-globin promoter up to and including nucleotide 127 upstream of the transcriptional start site and its derivatives containing six tandem copies of the synthetic SV40 B element enhancers oct$^+$sph$^+$ (B20 wt), oct$^+$sph$^-$ (B20 dpm2), oct$^-$sph$^+$ (B20 dpm7), and oct$^-$sph$^-$ (B20 dpm8) either 143 bp upstream (the pβΔ^{127} 6×B20/−143 series) or 2.2 kb downstream (the pβΔ^{127} 6×B20 series) of the β-globin initiation site have been described previously (Tanaka et al., 1988). The other β-globin reporter, pβΔ^{36}, contains the β-globin promoter sequence up to and including nucleotide 36 upstream of the transcriptional start site. This plasmid was constructed from pβe$^-$Δ (Baumruker et al., 1988) by point mutagenesis of the BamHI site (GGATCC to GGATCA) within the polylinker site upstream of the β-globin TATA box. The fragments containing six tandem copies of the synthetic enhancer were excised from the pβΔ^{127} downstream constructs by HindIII- and PstI-digestion and inserted into the SmaI site 52 bp upstream of the transcriptional start site in the pβΔ^{36} vector. The internal reference plasmid pα 4×(A+C) is a pUC119 derivative with the BamHI–PstI fragment containing the α-globin gene from πSVHPα2 (Treisman et al., 1983) and four tandem copies of the SV40 enhancer BstNI–PvuII fragment (nucleotides 232 to 270) containing the A and C enhancer elements, but not the octamer motifs, inserted downstream of the α-globin gene at the pUC119 SphI site.

Transfections and RNA Analysis

HeLa cells were transfected by the calcium phosphate coprecipitation procedure as described (Tanaka et al., 1988). The β-globin reporter plasmid (2 μg), internal reference pα 4×(A+C) (0.13 μg), effector plasmid (4 μg), and pUC119 DNAs (to 20 μg total DNA) were transfected into the cells on 100 mm dishes. Cytoplasmic RNAs were prepared by the NP40 lysis method and were analyzed by RNAase protection of mixed α- and β-globin antisense probes and electrophoresis through 6% denaturing polyacrylamide gels as described (Tanaka et al., 1988). The α-globin probe α132 and β-globin probe β350 are protected by the correctly initiated α-globin and β-globin transcripts over 132 and 350 nucleotides, respectively. Results were quantitated by densitometric analysis of selected autoradiograms. The activation assay for each construct described here was performed three or more times with similar results.

In Vitro Transcription and Translation

Templates for T7 in vitro transcription were made by the polymerase

pCG or PCGN expression constructs using as an upstream primer TTAATACGACTCACTATAGGGCGTGAAACTCCCGCA, which contains the T7 promoter sequence and can anneal to the HSV tk 5′ untranslated leader sequence, and as a downstream primer CCAAACTCACCCTGAAG, which can anneal to the rabbit β-globin sequences. The RNA templates for in vitro translation reaction were generated by in vitro transcription using these amplified products as templates for T7 RNA polymerase. The in vitro translations in rabbit reticulocyte lysates were carried out according to the manufacturer's recommendation (Promega). All the Oct-1 and Oct-2 wild-type and mutant proteins were in vitro translated and assayed for DNA binding activity by gel retardation. Except for the homeodomain mutant Oct-1H$^-$, all the Oct

Immunoprecipitation and Phosphatase Treatment

HeLa cells were transfected with 4 μg of pCGN constructs and, after incubation at 37°C for 40 hr, the cells were metabolically labeled with either 0.4 mCi of [^{35}S]methionine–cysteine mixture (Tran^{35}Slabel purchased from ICN) in methionine-free media or 1 mCi of [^{32}P]phosphoric acid in phosphate-free media for 4 hr. Whole-cell lysates were prepared by sonication followed by boiling for 3 min in RIPA buffer (0.5% NP40, 0.5% Tween 20, 0.5% deoxycholic acid, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, 20 mM Tris–HCl [pH7.5]) supplemented with 1% SDS, and the samples were subsequently diluted 10-fold in RIPA buffer without SDS. After preclearing the lysates with protein A–Sepharose, immunoprecipitates were prepared with 12CA5 anitibody (Niman et al., 1983; Field et al., 1988) and analyzed by electrophoresis through 8% SDS–polyacrylamide gels (Laemmli, 1970). For phosphatase treatment, immunoprecipitates were prepared in the same way, except that the lysates were prepared in the absence of SDS and without boiling. The immunoprecipitates were washed twice with 0.1 M MES buffer (pH

6.0) and suspended in 35 μl of 0.1 M MES buffer with or without potato acid phosphatase and 0.2 M sodium phosphate as specified. Portions were incubated at 37°C for 30 min, washed once with 0.1 M MES buffer containing 0.2 M sodium phosphate, washed twice with RIPA buffer, and analyzed on 8% SDS–polyacrylamide gels.

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