2000

Identification of the Transactivation Domain of the Transcription Factor Sox-2 and an Associated Co-activator

Tamara K. Nowling
University of Nebraska Medical Center

Lance R. Johnson
Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center

Matthew S. Wiebe
University of Nebraska-Lincoln, mwiebe2@unl.edu

Angie Rizzino
University of Nebraska Medical Center, arizzino@unmc.edu

Published in *Journal of Biological Chemistry* 275:6 (2000), pp. 3810–3818; doi: 10.1074/jbc.275.6.3810.

Submitted October 14, 1999; published February 11, 2000.

Identification of the Transactivation Domain of the Transcription Factor Sox-2 and an Associated Co-activator

Tamara K. Nowling,[1] Lance R. Johnson,[1,2] Matthew S. Wiebe,[1,2]

and Angie Rizzino[1,2]

1. Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198
2. Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198

Corresponding author – Angie Rizzino, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805; phone 402-559-6338, email arizzino@unmc.edu

Abstract

The importance of interactions between Sox and POU transcription factors in the regulation of gene expression is becoming increasingly apparent. Recently, many examples of the involvement of Sox-POU partnerships in transcription have been discovered, including a partnership between Sox-2 and Oct-3. Little is known about the mechanisms by which these factors modulate transcription. To better understand the molecular interactions involved, we mapped the location of the transactivation domain of Sox-2. This was done in the context of its interaction with Oct-3, as well as its ability to transactivate as a fusion protein linked to the DNA-binding domain of Gal4. Both approaches demonstrated that Sox-2 contains a transactivation domain in its C-terminal half, containing a serine-rich region and the C terminus. We also determined that the viral oncoprotein E1a inhibits the ability of the Gal4/Sox-2 fusion protein to transactivate, as well as the transcriptional activation mediated by the combined action of Sox-2 and Oct-3. In contrast, a mutant form of E1a, unable to bind p300, lacks both of these effects. Importantly, we determined that p300 overcomes the inhibitory effects of E1a in both assays. Together, these findings suggest that Sox-2 mediates its effects, at least in part, through the co-activator p300.

Transcription factors belonging to the Sox family contain a conserved HMG[1] DNA-binding domain that exhibits sequence-specific binding. At least 24 members of the Sox family expressed in many different cells and tissues have been identified (1). Members of the Sox family are further subdivided into subgroups based on the amount of homology outside of the HMG domain, as well as identity within the HMG domain. Unlike most transcription factors, Sox proteins bind DNA in the minor groove. Hence, Sox factors are able to bind DNA in close proximity to transcription factors that bind in the major groove. Indeed, interaction and/or cooperation between Sox factors and other transcription factors has been observed, including Sox-10 with Tst-1/Oct6/SCIP (2), Sox-11 and Sox-4 with Brn-1 (3), and Sox-2 with Oct-3 (4). Sox and POU transcription factors, such as Sox-2 and Oct-3, are co-expressed in many different cell types during development (1, 5, 6), as well as in embryonal carcinoma (EC) and embryonic stem cells. Examples of the involvement of Sox-POU partnerships in transcriptional regulation include transcription of the *FGF-4*, *Osteopontin* (*OPN*), and *UTF1* genes in EC cells (4, 7, 8). Recently, the interaction between Sox-2 and Oct-3 has been linked closely to the transcription of the *FGF-4* gene.

Expression of the *FGF-4* gene is controlled by an essential enhancer located in the untranslated region of the third exon, 3 kilobases downstream of the transcription start site (9–11). This distal enhancer contains three important motifs: a POU motif, which binds several octamer-binding proteins (11–14); a HMG motif, which binds several members of the Sox transcription factor family (3, 15, 16); and a GC box, which binds Sp1 and Sp3 (14). The functional importance of these three binding sites for the transcription of the *FGF-4* gene has been demonstrated through site-directed mutagenesis studies (11, 13).[2] The factors that are functionally active at the HMG and POU motifs are Sox-2 and Oct-3, respectively (11, 15, 16). *Sox-2* and *Oct-3* are co-expressed with the *FGF-4* gene in the preimplantation mouse embryo (1, 17, 18) and in F9 EC and embryonic stem cells (12) but are down-regulated upon differentiation of these cells (19–21). Furthermore, the HMG and POU sites are separated by only three base pairs, and inserting five base pairs between these sites results in a loss of enhancer function (4).[3] Synergism and physical interaction between Sox-2 and Oct-3 at their binding sites has been demonstrated (4), indicating that the *FGF-4* gene is likely to be regulated by the concerted action of Sox-2 and Oct-3.

The *UTF1* and *OPN* genes are also co-expressed with *Sox-2* and *Oct-3* in F9 EC and embryonic stem cells, are down-regulated upon differentiation, and contain an HMG motif and a POU motif (7, 8). In the *UTF1* gene, the HMG and POU motifs are located next to one another and approximately 1.8 kilobases downstream of the transcription start site, similar to the *FGF-4* gene (7). In contrast, these motifs are separated by 48 base pairs and are located relatively close to the transcription start site in the *OPN* gene (8). Both genes are regulated by Sox-2 and Oct-3. However, the *UTF1* gene is regulated by the synergistic action of Sox-2 and Oct-3 (7), whereas Sox-2 represses the Oct-3 activation of the *OPN* gene (8). The repression of the *OPN* gene by Sox-2 requires the C-terminal region of Sox-2. Positive regulation of the *FGF-4* and *UTF1* genes and negative regulation of the *OPN* gene by the combination of Sox-2 and Oct-3 may be attributed to the arrangement of the HMG and POU motifs relative to each other, as well as to other transcription factor binding sites. These studies illustrate that the effects of Sox factors on transcription are dependent on the

context of the gene, but little is known about the mechanisms that Sox factors use to modulate transcription.

Recently, it has been reported that the transactivation domain of several Sox family members is located in the C-terminal half of the molecule (3, 22–24). However, it is unclear whether more than one region of the molecule is needed for transactivation, nor is it clear whether the domains involved are dependent on transcription factors that act in concert with Sox proteins. Transactivation by many transcription factors requires the action of a co-activator. In the context of the HMG and POU sites of the *FGF-4* gene, Sox-2 and Oct-3 activate transcription from a distance. Therefore, co-factors are likely to be involved in bridging these proteins to the transcriptional machinery. Interestingly, E1a has been shown to repress the Oct-3-mediated expression of the *Rex-1* gene (25), as well as the transcription of several other genes (reviewed in Ref. 26). In many cases, overexpression of the co-factor p300, a ubiquitous protein that can interact directly with E1a, was able to overcome E1a inhibition (27–31), indicating a possible role for p300 as a co-activator.

To advance our understanding of the molecular mechanisms by which Sox-2 acts, several questions are addressed in this study. First, which domains of Sox-2 are necessary for transactivation? Second, does Sox-2 on its own or in combination with Oct-3 mediate its effects through a co-activator? Here, we show that the C-terminal half of Sox-2, including its serine-rich domain and extreme C-terminal end, is necessary for transactivation. We also demonstrate that E1a inhibits the ability of a Gal4/Sox-2 fusion protein to transactivate, as well as the transcriptional activation mediated by Sox-2 and Oct-3. Importantly, we demonstrate that the co-activator p300 can recover this inhibition by E1a. These findings are the first to implicate p300 in the action of the Sox family of transcription factors.

Experimental Procedures

Cell Culture and Transient Transfections

HeLa cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone). HeLa cells were seeded at 5×10^5 cells per 100-mm dish unless otherwise noted for 24 h prior to transfection. Cells were transfected by the calcium phosphate precipitation method, as described previously (11, 32), incubated with the precipitate for 14–16 h, and refed with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were harvested the following day. Cell extracts were prepared, and chloramphenicol acetyltransferase (CAT) and β-galactosidase activities were determined, as reported previously (16). The total amount of transfected DNA was kept constant by addition of null vectors. To adjust for transfection efficiency, all transfections were normalized with either pCH110 (SV40β-gal) (Amersham Pharmacia Biotech) or pCMVβ-gal (CLONTECH) as indicated in the figure legends. All transfections were performed in duplicate or triplicate with representative transfections shown. Plasmid DNA was purified by Qiagen tip-500 columns.

Promoter-Reporter and Sox-2 Deletion Constructs

The promoter-reporter construct pCatSO3 was constructed as follows: complimentary oligonucleotides (synthesized by the Eppley Cancer Institute Molecular Biology Core Facility) containing two sets of the Sox-2 and Oct-3 binding sites (in boldface) from the FGF-4 enhancer region and *Bgl*II sites at both ends (5′-GATCT**CTTTGTT**TGG**ATGCTAAT**GGC T**CTTTGTT**TGG**ATGCTAAT**GGA-3′) were annealed and ligated to generate multimers. These multimers were then ligated into a *Bgl*II site upstream of the SV40 promoter of the pCat vector (Promega) to generate pCatSO3, which contains a total of six tandem copies of the HMG and POU sites. pCMVSox-2 3′ deletion constructs were generated by digesting pCMVSox-2 (16) at the *Not*I site 5′ of the Sox-2 insert and *Acc*I, *Sty*I, or *Aat*II sites within the 3′ end of the Sox-2 sequence. The Sox-2 fragments *Not*I-*Acc*I, *Not*I-*Sty*I, and *Not*I-*Aat*II were isolated and ligated into pCMVΔSox-2N/X vector (pCMVSox-2 digested at the *Not*I and *Xho*I sites flanking the Sox-2 sequence and isolated), to generate pCMVSox-$2_{1–293}$, pCMVSox-$2_{1–242}$, and pCMVSox-$2_{1–202}$, respectively. pCMVSox-$2_{1–180}$ (pCMVNP2) has been described previously (16).

Sox-2 was isolated from pCMVSox-2 by digesting with *Not*I and *Xho*I and then cloned into pCR-Script Amp SK(+) (Stratagene) to generate pCRSox-2. This construct was then used to generate additional Sox-2 deletions. pCRSox-$2_{\Delta177–293}$ was generated by performing PCR with pCRSox-2 and the 3′ primer 177L (5′-CTGCTCCTGCATCATGCTGTAGCTG-3′) and the 5′ primer 293U (5′-CGACTGCACATGGCCCAGCTC-3′). This amplified the entire plasmid excluding the region of Sox-2 of base pairs 537–879 (amino acids 177–293). The PCR product was then digested with *Dpn*I, which will digest methylated DNA destroying the parental plasmid, ligated, and transformed into bacteria. Similarly, pCRSox-2 and either the primer pair of 3′ primer 45L (5′-CCTCTTGACGCGTTCCGGGCT-3′) and 5′ primer 116U (5′-ATCCGGAAAACCAAGACGCTCATGAAG-3′) or the primer pair of 3′ primer 207L (5′-CTGCAGGGCGCTGACGTCGTA-3′) and 5′ primer 254U (5′-GATCCCGTGGTTA CCTCTTCCTCCCA-3′) were used in PCRs to generate pCRSox-$2_{\Delta45–116}$ and pCRSox-$2_{\Delta207–254}$, respectively. pCRSox-$2_{\Delta45–116}$ excludes the region of Sox-2 of base pairs 135–348 (amino acids 45–116) and pCRSox-$2_{\Delta207–254}$ excludes the region of Sox-2 of base pairs 621–762 (amino acids 207–254). In the case of the Δ45–116 and Δ207–254 constructs, an additional three base pairs were added to the 5′ end of the 5′ primers, which, after PCR and ligation, generated a *Bam*HI site that was used for screening. After confirmation by sequencing, the Sox-2 deletions were isolated following *Not*I-*Xho*I digestion and ligated into pCMVΔSox-2N/X.

The 5′ end of Sox-2 was tagged with the Flag epitope as follows. A 5′ primer (5′-CGCGCGGGTACCGCCACCATGGACTACAAGGACGACGATGACATGTATAACATG ATGGAGACG-3′) containing a *Kpn*I site, a Kozac sequence, the Flag sequence, and 24 base pairs of the 5′ Sox-2 sequence and the 3′ primer 45L (described above) were used in a PCR with pCMVSox-2 as template. The resulting product was digested with *Kpn*I and *Hin*dIII and ligated into pCMVΔSox-2K/H vector (pCMVSox-2 digested with *Kpn*I and *Hin*dIII and isolated), generating pCMVFlagSox-2. The same method was used to insert the Flag sequence into the pCMVSox-2 deletion constructs. Sequencing confirmed that all constructs contained the Flag sequence in-frame with Sox-2. A GFPSox-2 expression vector was constructed by ligating a *Sac*I-*Kpn*I Sox-2 fragment from pCRSox-2 into the *Sac*I-*Kpn*I sites of

pEGFP-C1 (CLONTECH), fusing green fluorescent protein (GFP) in-frame to the N terminus of Sox-2. Positive clones were verified by sequencing.

Other plasmids utilized included pCMVOct-3 (11); Gal4/Sox-11 C3, obtained from Michael Wegner (3); pCMVOct-1, obtained from Clinton Jones (University of Nebraska, Lincoln, NE); pCMV12S E1a and pCMV12S E1aΔ2–36 (mE1a), a mutant E1a unable to bind p300, obtained from Joseph Nevins (33); and pCMVp300 and pCMVp300del30, a mutant p300 unable to bind E1a, obtained from David Livingston (34).

Gal4 Constructs

Gal4/Sox-2 fusions were constructed by ligating PCR-generated Sox-2 fragments into the *Eco*RI and *Hin*dIII sites of the SV40Gal4 fusion vector, pM3, obtained from Ivan Sadowski (35). The following primers were used in PCRs to generate the Sox-2 fragments indicated: Sox-2121–319, 121U (5′-CCGTCAATGAATTCTCATGAAGAAGGATAAGTACACG-3′) and 319L (5′-TATTTGAAGCTTCTCACATGTGCGACAGGGG-3′); Sox-2206–319, 206U (5′-GTCAATGAATTCTGCAGTACAACTCCATGACCAGC-3′) and 319L; Sox-2206–291, 206U and 291L (5′-TATTTGAAGCTTGGGCGCAGCGGGCT-3′); Sox-2206–260, 206U and 260L (5′-TATTTGAAGCTTGGAGGAAGAGGTAACCACGGG-3′); Sox-2291–319, 291L (5′-GTCAATGAATTCCCAGTAGACTGCACATGGCC-3′) and 319L. All resulting constructs were sequenced and determined to be in frame with Gal4 and named as indicated in figure 3*A*. These constructs were co-transfected with pG5EC obtained from Ivan Sadowski (35). pG5EC contains five Gal4 DNA-binding sites upstream of the TATA box from the adenovirus E1b gene driving the CAT reporter gene. *Bgl*II-*Hin*dIII fragments from pM3 and Gal4/Sox-2206–319 were ligated into the *Bgl*II and *Hin*dIII sites of pCMV5 (36) to generate the plasmids CMVGal4 and CMVGal4/Sox-2206–319, respectively.

Western Blotting

HeLa cells were transfected as described above. In each case, CMVβ-gal was co-transfected with the plasmids indicated in the figure legends and used to normalize for differences in transfection efficiency. Whole cell extracts were prepared after transfection as follows. Cells were washed twice with 5 ml of cold phosphate-buffered saline, harvested into 1 ml of cold phosphate-buffered saline, and centrifuged. The cell pellets were resuspended in lysis buffer (10 mM Tris, pH 7.4–8.0, 150 mM NaCl, 1% Triton X-100, various protease and phosphatase inhibitors) and incubated on ice for 30 min. Following centrifugation, the supernatant was transferred to a new tube, aliquoted, and stored at –80°C. Extracts were assayed for β-galactosidase activity, normalized, and diluted in 4× sample buffer (250 mM Tris-HCl, pH 6.8, 8% w/v SDS, 40% glycerol, 200 mM dithiothreitol, 0.4% (w/v) bromphenol blue), heated to 95–100°C for 5 min, and loaded onto a Tris-glycine gel. Gels were subjected to electrophoresis in running buffer (25 mM Tris, 0.2 M glycine, and 0.1% SDS). Proteins were then transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore) in transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol) and membranes were blocked in a 3% bovine serum albumin solution. For detection of the Gal4 fusion proteins, membranes were incubated with an anti-Gal4 DNA-binding domain (DBD) polyclonal antibody (Upstate Biotechnology, Lake Placid, New York) diluted 1:2000 in blocking buffer +0.1% Tween-20, washed in TBS/T (Tris buffered saline, 0.1% Tween-20), and incubated

with an alkaline phosphatase-conjugated secondary antibody (Pierce) diluted 1:10 000 in TBS/T. For detection of the Flag fusion proteins, membranes were incubated with an anti-Flag M2 monoclonal antibody (Sigma) diluted 1:2000 in TBS/T, washed in TBS/T, and incubated with an alkaline phosphatase-conjugated secondary antibody (Pierce) diluted 1:2000 in TBS/T. Proteins were detected using the enhanced chemifluorescence (ECF) kit (Amersham Pharmacia Biotech) and scanned on a Storm phosphorimager (Molecular Dynamics). Quantitation was performed using the ImageQuant analysis software (Molecular Dynamics). All Western blots were performed in duplicate or triplicate, with representative Western blots shown.

Results

Mapping of the Sox-2 Transactivation Domains Using the Gal4 System

The HMG and POU sites located in the enhancer of the *FGF-4* gene are crucial for transcription (9, 11, 13). Furthermore, overexpression studies have shown that Oct-3 on its own can stimulate transcription of promoter-reporter constructs containing the HMG and POU sites of the *FGF-4* enhancer (11) and that the addition of Sox-2 results in a synergistic stimulation of transcription (15). Although the transactivation domains of Oct-3 have been mapped (37–40) and Sox-2 and Oct-3 have been shown to physically interact (4), it is unclear how these factors cooperate to stimulate transcription. Therefore, to advance our understanding of this partnership, we set out to investigate the molecular mechanisms by which Sox-2 acts by mapping the transactivation domain of Sox-2. Previous studies demonstrated that the N-terminal region of Sox-2 (Sox-$2_{1–180}$) contains the DNA-binding domain (15, 16). These studies and others (3, 22, 23) indicate that the transactivation domain of Sox-2 is likely to be located in the C-terminal half of the protein.

To map the domains of Sox-2 transactivating activity, a series of fusion proteins containing the DBD of Gal4 and various regions of Sox-2 was constructed. Gal4 fusion proteins have been used widely to identify transactivation domains of transcription factors. In these studies, various Sox-2 C-terminal regions were cloned into pM3, a Gal4 DBD expression vector (Fig. 1*A*). These Gal4/Sox-2 fusion constructs were cotransfected into HeLa cells with the promoter-reporter CAT construct pG5EC, containing five copies of the Gal4 binding site. The region from just C-terminal of the HMG domain to the C-terminal end (amino acids 121–319) exhibited maximum transactivation at the concentration indicated (fig. 1*B*). However, the region from the serine-rich domain to the C-terminal end, amino acids 206–319, also contains considerable transactivation potential (fig. 1*B*). Constructs containing only the serine-rich domain (Gal4/Sox-$2_{206–291}$ and Gal4/Sox-$2_{206–260}$) or the last 28 amino acids of the C terminus (Gal4/Sox-$2_{291–319}$) failed to transactivate, whereas constructs containing both of these domains (Gal4/Sox-$2_{121–319}$ and Gal4/Sox-$2_{206–319}$) were able to transactivate. This suggests that both the serine-rich domain (amino acids 206–260) and the C-terminal end (amino acids 260–319) are necessary for transactivation. A Gal4/Sox-11 construct (Gal4/Sox-11 C3) (3) served as a positive control and was able to transactivate in all of the Gal4 experiments (data not shown).

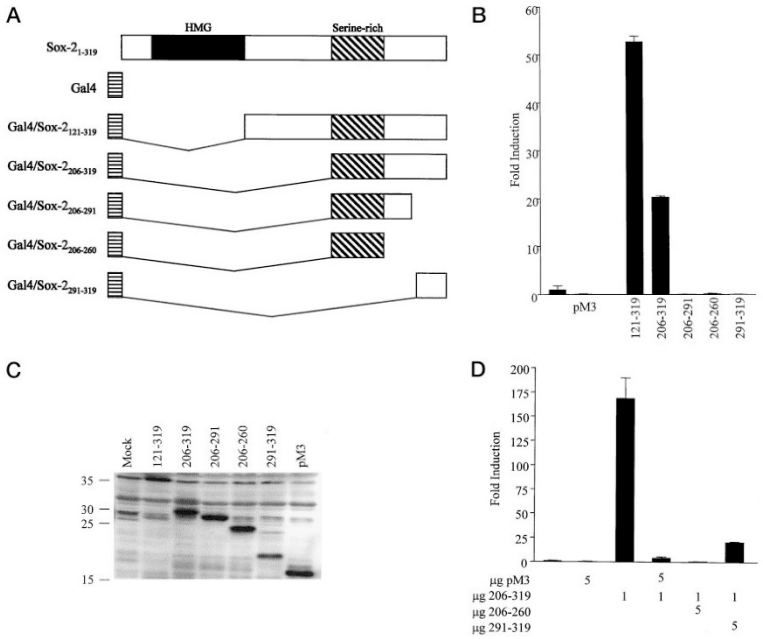

Figure 1. *Mapping of the transactivation domain of Sox-2. A,* schematic representation of the fusions between the Gal4 DBD (Gal4) driven by a SV40 promoter and various regions of murine Sox-2 as described under "Experimental Procedures." *Subscript numbers* indicate the amino acids of Sox-2 contained in the fusion proteins. *B,* transfection into HeLa cells with 2 μg of the promoter-reporter construct pG5EC alone or in combination with either 5 μg of pM3 or 1 μg of the indicated Gal4/Sox-2 plasmids. *C,* expression of Gal4 fusion proteins in transfected HeLa cells. Extracts from transfected cells were subjected to Western blot analysis with an antibody to the Gal4 DBD. Fusion proteins were detected by ECF. The predicted sizes of the Gal4/Sox-2 fusion proteins are as follows: 121–319, 38 kDa; 206–319, 28.6 kDa; 206–291, 25.5 kDa; 206–260, 22.1 kDa; 291–319, 19.3 kDa; and Gal4 (pM3), 16.2 kDa. Molecular mass markers (in kDa) are shown on the *left*. *D,* transfection into HeLa cells with 2 μg of pG5EC alone or in combination with either 5 μg of pM3 or 1 μg of Gal4/Sox-2206–319 plus 5 μg of the Gal4/Sox-2 plasmids indicated. pCMVb-gal was used for normalization in all transfections. A constant amount of total DNA was achieved by adding a null SV40-expressing plasmid. Results of transfections are presented as fold induction over the expression of pG5EC alone. pG5EC is a promoter-reporter plasmid containing five copies of the Gal4 DNA-binding site upstream of a SV40 promoter driving the CAT gene, and pM3 is a Gal4 DBD expression vector driven by a SV40 promoter.

Western blot analysis demonstrated that the Gal4/Sox-2 fusion proteins were expressed (fig. 1*C*). Although a number of nonspecific bands were detected due to the amount of protein loaded, it is evident that each of the Gal4/Sox-2 fusion proteins was expressed at the predicted size (see the legend to fig. 1). The slight differences in expression levels do not correlate with transactivation levels and therefore do not explain the failure of several Gal4/Sox-2 fusion proteins (Gal4/Sox-2$_{206–291}$, Gal4/Sox-2$_{206–260}$, and Gal4/Sox-2$_{291–319}$) to transactivate (compare fig. 1*B* and fig. 1*C*). Furthermore, each of the inactive constructs, as well as Gal4, when titrated against Gal4/Sox-2$_{206–319}$ in co-transfection assays, drastically

inhibited the transactivation observed with Gal4/Sox-$2_{206–319}$ (fig. 1*D* and data not shown). Together, these results argue strongly that the inactive constructs are expressed and are able to compete for binding to the Gal4 binding sites.

Deletion Mapping of the Sox-2 Transactivation Domains

To map the transactivation domains of Sox-2 in the context of its synergistic stimulation of transcription in conjunction with Oct-3, HeLa cells were utilized because they do not contain endogenous Sox-2 or Oct-3. In addition, a promoter-reporter construct, pCatSO3, was designed that contains six copies of the HMG and POU motifs from the *FGF-4* enhancer upstream of a SV40 promoter driving the CAT gene (fig. 2*A*). In HeLa cells, pCatSO3 has minimal activity on its own and relatively little activity when co-transfected with either a Sox-2 or an Oct-3 expression vector (fig. 2*B*). In contrast, a substantial stimulation was observed when pCatSO3 was co-transfected with both Sox-2 and Oct-3 expression vectors (fig. 2, *B* and *C*), illustrating the synergistic interaction of Sox-2 and Oct-3. Additionally, a truncated Sox-2 protein, Sox-$2_{1–180}$, which retains its DNA-binding and Oct-3 interacting domain, did not stimulate pCatSO3 on its own but did induce some stimulation in conjunction with Oct-3. However, the stimulation observed with Sox-$2_{1–180}$ and Oct-3 was significantly less than that observed with full-length Sox-2 (Sox-$2_{1–319}$) and Oct-3 (fig. 2, *B* and *C*). This latter finding suggests that Sox-2 transactivation is dependent, at least in part, on its C-terminal region.

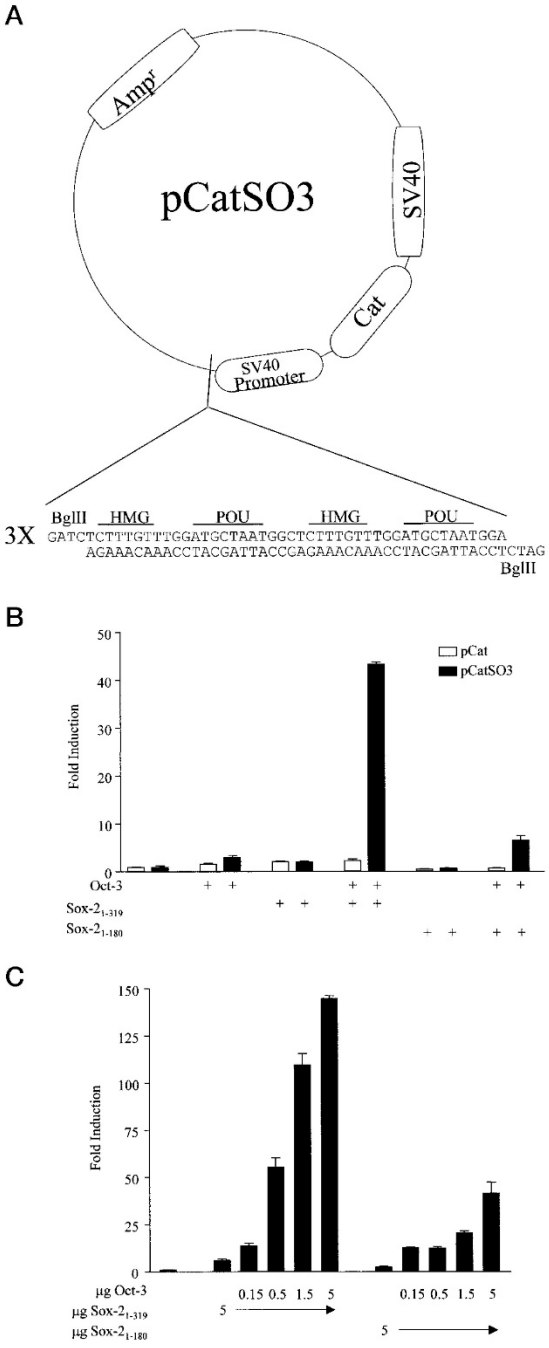

Figure 2. *Expression and activation of the promoter-reporter construct pCatSO3 in HeLa cells. A,* physical map showing the construction of the pCatSO3 vector. Three copies of annealed oligonucleotides containing two sets of the HMG and POU sites of the FGF-4 enhancer were ligated into a *Bgl*II site upstream of the SV40 promoter driving the CAT reporter gene as described under "Experimental Procedures." *B,* cells were transfected with 5 µg

of the parent vector pCat alone or in combination with 1 μg of the CMV-expressing plasmids indicated or with 5 μg of pCatSO3 alone or in combination with 1 μg of the CMV-expressing plasmids indicated. *C*, cells were transfected with 5 μg of pCatSO3 alone or in combination with the amount of CMV-expressing plasmids indicated. pCH110 (SV40β-gal) was used for normalization in all transfections. A constant amount of total DNA was achieved by adding the null CMV vector, pCMV5. *Subscript numbers* indicate the amino acids of Sox-2 contained in the construct. Results of transfections are presented as fold induction over the expression of pCat (*open bars*) or pCatSO3 (*solid bars*) alone.

To further map the transactivation domains of Sox-2 in the context of the HMG and POU motifs of the *FGF-4* enhancer, a series of CMV expression vectors containing Sox-2 C-terminal deletions was constructed (fig. 3*A*). These constructs were co-transfected with pCatSO3 into HeLa cells with or without Oct-3. None of the Sox-2 deletions were able to transactivate on their own, but all were able to stimulate transcription in conjunction with Oct-3 (fig. 3*B*). However, the stimulation observed by Oct-3 and the Sox-2 deletion mutants was reduced significantly compared with the stimulation with Oct-3 and Sox-$2_{1–319}$ (fig. 3*B*). In all cases, the stimulation was reduced approximately to the level of that observed with the shortest Sox-2 construct, Sox-$2_{1–180}$, which indicated that deleting even a small region from the C terminus (amino acids 293–319) results in a significant reduction in transactivation. However, because the truncated proteins have little activity on their own compared with Sox-$2_{1–319}$ (fig. 3*B*), the transactivation domain of Sox-2 is not likely to be located in the N-terminal region. To determine whether regions other than the extreme C terminus (amino acids 293–319) were necessary for transactivation, Sox-2 mutants containing internal deletions of amino acids 207–254 (Sox-$2_{\Delta 207–254}$) or 177–293 (Sox-$2_{\Delta 177–293}$) were constructed. Transfections with Oct-3 and these Sox-2 mutants resulted in decreased stimulation of pCatSO3 (fig. 3*C*). These results indicate that regions other than the extreme C terminus are necessary for transactivation. A construct containing a deletion of the HMG domain (Sox-$2_{\Delta 45–116}$) results in no additional stimulation above that seen with Oct-3 alone. This was expected because the HMG domain is necessary for binding to the HMG motif (15, 16) and for interaction with Oct-3 (4).

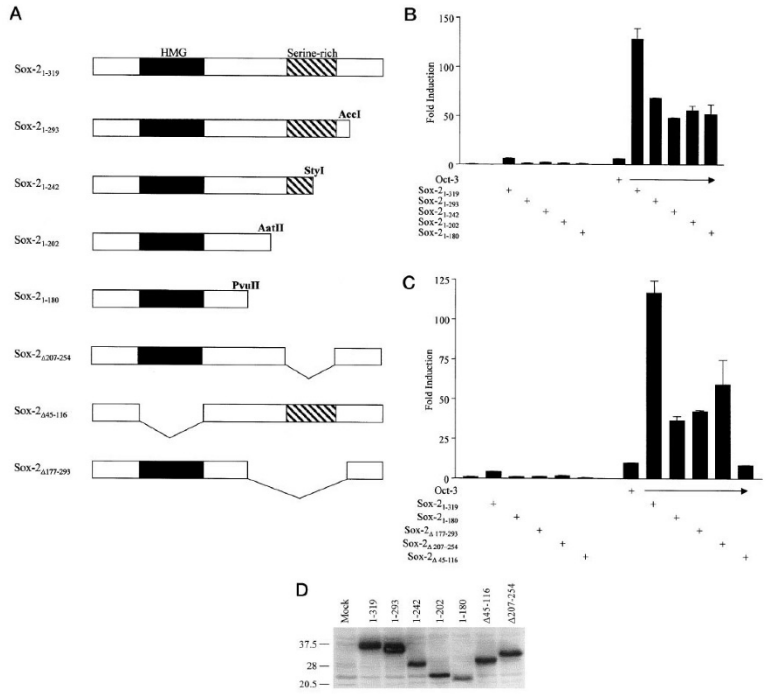

Figure 3. *Deletion mapping of the Sox-2 transactivation domain. A,* schematic representation of Sox-2 and the Sox-2 deletion cDNAs. All deletions were ligated into a CMV expression plasmid as described under "Experimental Procedures." *B,* HeLa cells were transfected with 5 μg of pCatSO3 alone or in combination with 1 μg of the CMV-expressing plasmids indicated. *C,* HeLa cells were transfected with 5 μg of pCatSO3 alone or in combination with 1 μg of the CMV-expressing plasmids indicated. pCH110 or CMVb-gal was used for normalization. A constant amount of total DNA was achieved by adding the null CMV-expressing vector, pCMV5. Results of transfections are presented as fold induction over the expression of pCatSO3 alone. *D,* expression of FlagSox-2 fusion protein and FlagSox-2 deletion fusion proteins in transfected cells. Extracts from transfected HeLa cells were subjected to Western blot analysis with an antibody to Flag. Fusion proteins were detected by ECF. The predicted sizes of the FlagSox-2 proteins are as follows: 1–319, 37.7 kDa; 1–293, 34.8 kDa; 1–242, 29.2 kDa; 1–202, 24.8 kDa; 1–180, 22.4 kDa; D207–254, 32.5 kDa; and D45–116, 29.9 kDa. Molecular mass markers (in kDa) are shown on the *left*.

To ensure that these Sox-2 deletions were being expressed, the Flag sequence was inserted at the N terminus of Sox-2$_{1–319}$ and all of the Sox-2 deletion constructs. Extracts from cells transfected with these constructs were subjected to Western blot analysis with anti-Flag M2 antibody. Protein expression from the FlagSox-2 deletion constructs at the predicted sizes was observed readily (fig. 3*D*). Although small differences in the expression levels from several of these constructs was observed, their protein expression levels do not account for their activity levels. Furthermore, the Flag epitope did not alter the function of these constructs because all of the FlagSox-2 deletion constructs were able to stimulate transcription in conjunction with Oct-3 (data not shown) similar to that observed with the

untagged deletion constructs (fig. 3*B*). Because all of the above Sox-2 deletion constructs contain the DNA-binding (HMG) and Oct-3 interaction domain, the observed stimulation in conjunction with Oct-3 may be due to a stabilization of Oct-3 binding (see under "Discussion").

p300 Recovers E1a Inhibition of pCatSO3 and Stimulates the Activity of Sox-2/Oct-3

Previous results have shown that E1a inhibits the synergistic activation of the *Rex-1* gene by Rox-1 and Oct-3 (25). The Rox-1 and POU sites are adjacent to each other on the *Rex-1* promoter, similar to the HMG and POU sites of the *FGF-4* enhancer. Furthermore, E1a reduces the expression of *FGF-4* promoter-reporter constructs containing the HMG and POU motifs that bind Sox-2 and Oct-3, respectively.[4] Therefore, the effect of E1a on Sox-2/Oct-3 activation of pCatSO3 was examined. A dose-dependent decrease of transcriptional activity was observed (fig. 4*A*). In contrast, a mutant form of E1a (mE1a) that lacks amino acids 2–36 and that does not bind p300 had little effect on Sox-2/Oct-3 activation of pCatSO3 (fig. 4*A*). Control transfections with pCatSO3, pCMVβ-gal, or pCMVCat demonstrated that neither E1a nor mE1a has any effect on the basal expression of pCatSO3 or on the expression of the CMV promoter, which drives several reporter genes (data not shown, also see fig. 5*C*). This suggested a role for p300 in mediating the effects of Sox-2/Oct-3. To examine this possibility, HeLa cells were transfected with pCatSO3 and with Oct-3, Sox-2, and p300 expression vectors with or without an E1a expression vector. We observed that p300 was able to recover, in a dose-dependent manner, the inhibition by E1a (fig. 4*B*). Additionally, p300 seemed to further stimulate the transcription of pCatSO3 by Sox-2 and Oct-3 (fig. 4*B*). Interestingly, a p300 construct mutant for binding to E1a (p300del30) also was able to recover the E1a inhibition (fig. 4*B*). Extracts from cells transfected as above, but with the FlagSox-2 construct, were subjected to Western blot analysis with anti-Flag M2 antibody. Neither E1a nor p300 alone or in combination affected the level of FlagSox-2 protein (fig. 4*C*), and the Flag epitope did not alter the function of this construct (data not shown). These results indicate that p300 can mediate the transcriptional effects of Sox-2 and Oct-3.

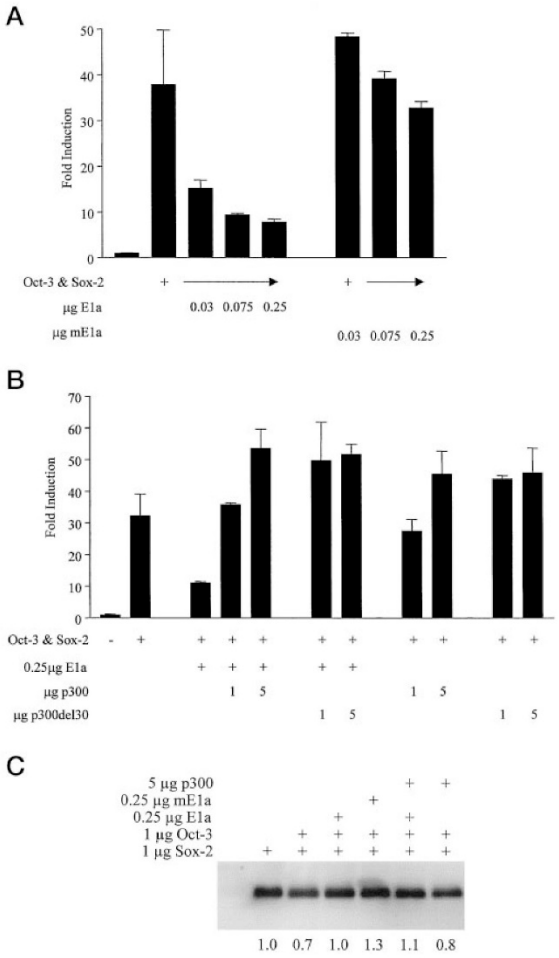

Figure 4. *Effects of E1a and p300 on activation of pCatSO3 by Sox-2 and Oct-3. A,* transfection of HeLa cells with 5 µg of pCatSO3 alone or in combination with 1 µg each of the CMV expression plasmids Sox-2 and Oct-3 plus increasing amounts of CMV plasmids expressing 12S E1a (*E1a*) or 12S E1aΔ2–36 (*mE1a*), as indicated. *B,* transfection of HeLa cells with 5 µg of pCatSO3 alone or in combination with 1 µg each of the CMV-expressing plasmids Sox-2 and Oct-3 along with the amount of CMV-expressing plasmids indicated. A constant amount of total DNA was achieved by adding the null CMV vector, pCMV5. Results are presented as fold induction over the expression of pCatSO3 alone. *C,* expression of FlagSox-2 fusion protein in HeLa cells transfected with FlagSox-2 alone or in combination with E1a, mE1a, and/or p300 CMV expression vectors. Extracts from transfected cells were subjected to Western blot analysis with an antibody to Flag. Fusion proteins were detected by ECF, and the resulting bands were quantitated. Expression values, relative to the expression of Sox-2 only, are shown below each lane. pCH110 or CMVb-gal was used for normalization. mE1a is a plasmid expressing a mutant E1a unable to bind p300.

To extend these findings, we examined the effect of E1a on the ability of Gal4/Sox-2 to transactivate and whether p300 is mediating the transcriptional effects of Sox-2. For these

studies, a Gal4/Sox-2 fusion construct driven by the CMV promoter was utilized. We observed that E1a, but not mE1a, inhibits the transactivation of the Gal4/Sox-2206–319 construct, which contains the serine-rich domain and C-terminal end (fig. 5*A*). As with the pCatSO3 construct, p300 was able to recover the E1a inhibition of Gal4/Sox-$2_{206–319}$ transactivation, as well as further stimulate the transactivation of Gal4/Sox-$2_{206–319}$ (fig. 5*B*). Furthermore, we demonstrated that E1a, mE1a, and p300 had no effect on the basal expression of the promoter-reporter construct, pG5EC (data not shown). Importantly, E1a did not have a significant effect on the protein levels of Gal4 or Gal4/ Sox-$2_{206–319}$ driven by the CMV promoter (fig. 5*C*). Therefore, a reduction in Gal4/Sox-$2_{206–319}$ fusion protein levels does not account for the substantial reduction in Sox-2 transactivation by E1a (compare fig. 5*B* to fig. 5*C*). As an added control, the effect of E1a on the nuclear localization of Sox-2 was examined by transfecting a GFPSox-2 expression vector alone or in combination with E1a into HeLa cells. We determined that E1a does not affect the localization of Sox-2 into the nucleus (fig. 6), nor does E1a affect GFP itself (data not shown). This indicates that E1a is not inhibiting the transactivation of Sox-2 by preventing Sox-2 from localizing to the nucleus. Together, these results suggest strongly that Sox-2 transactivation is mediated by the co-activator p300.

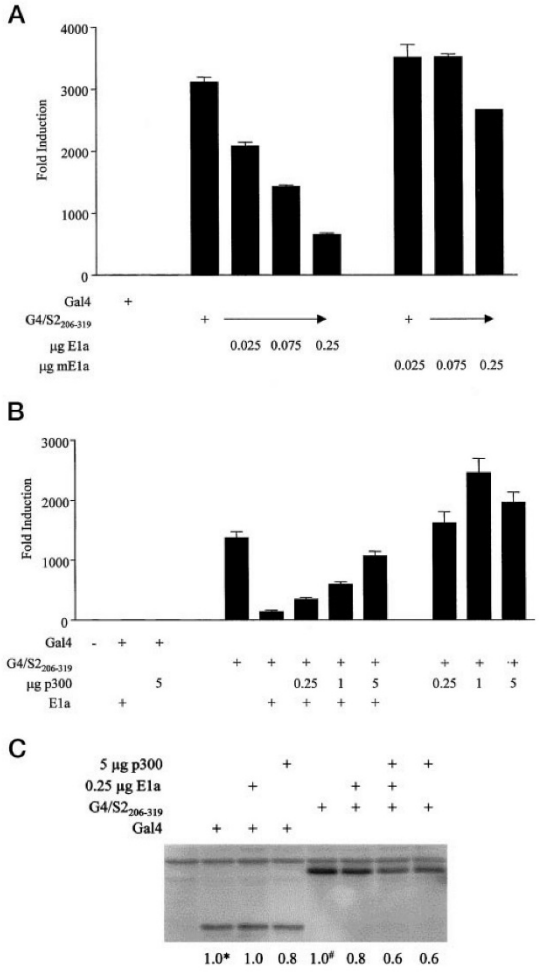

Figure 5. *Effects of E1a and p300 on Sox-2 transactivation. A,* transfection of HeLa cells with 2 μg of pG5EC alone or in combination with 1 μg of CMVGal4 or 1 μg of CMVGal4/Sox-2$_{206–319}$ (*G4/S2$_{206–319}$*) expression vectors and increasing amounts of the CMV plasmids expressing 12S E1a (*E1a*) or 12S E1aΔ2–36 (*mE1a*) as indicated. *B,* transfection of HeLa cells with CMVGal4/Sox-2$_{206–319}$ (*G4/S2$_{206–319}$*) alone or in combination with 0.25 μg of CMV plasmid expressing E1a and increasing amounts of the CMV plasmid expressing p300 indicated. A constant amount of total DNA was achieved by adding the null CMV vector, pCMV5. Results are presented as fold induction over the expression of pG5EC alone. *C,* expression of Gal4 protein and G4/S2$_{206–319}$ fusion protein in HeLa cells transfected with Gal4 or G4/S2$_{206–319}$ alone or in combination with CMV plasmids expressing E1a and/or p300. Extracts from transfected cells were subjected to Western blot analysis with anti-Gal4 DBD antibody. Fusion proteins were detected by ECF, and the resulting bands were quantitated. Expression values for Gal4 in the presence of E1a or p300, relative to the expression of Gal4 in the absence of E1a or p300 (*) and expression values for G4/S2$_{206–319}$ in the presence of E1a and/or p300, relative to the expression of G4/S2$_{206–319}$ in the absence of E1a or p300 (#), are shown below each lane. pCMV*β*-gal was used for normalization in all transfections. mE1a is a plasmid expressing a mutant E1a unable to bind p300.

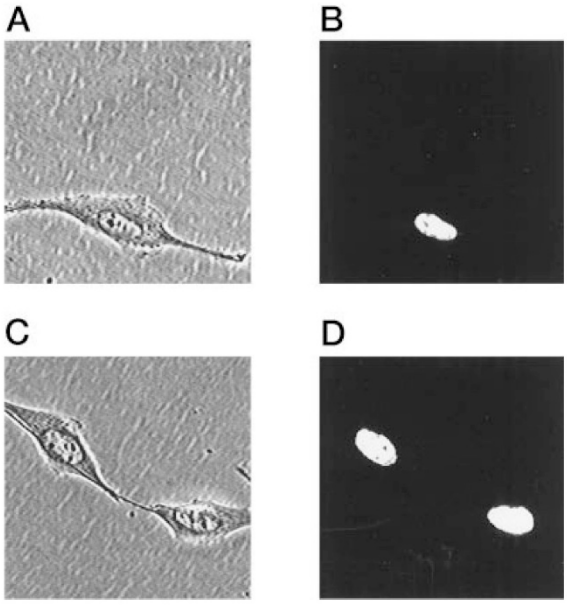

Figure 6. *Effect of E1a on the nuclear localization of Sox-2.* HeLa cells (10^5) were transfected with GFPSox-2 alone or in combination with CMV12S E1a. A representative field from each transfected plate is shown. *A* and *B,* cells transfected with 1 μg of GFPSox-2. *C* and *D,* cells transfected with 1 μg of GFPSox-2 and 0.25 μg of CMV12S E1a. Photomicrographs were taken under brightfield (*A* and *C*) and fluorescence (*B* and *D*).

Discussion

The Transactivation Domains of Sox-2 Include a Serine-rich Domain and the Extreme C-terminal End

The transactivation domains of several Sox factors, including Sox-11, 4, and 9, have been identified recently (3, 22, 23). In this study, we have mapped the location of the transactivation domain of the murine Sox-2 gene product in the context of two experimental promoters, including one that responds to the synergistic action of Sox-2 and Oct-3. For each of the Sox factors studied thus far, including Sox-2, the transactivation domain has been mapped to the C-terminal region. This is interesting, given that there is little sequence homology between the C-terminal region of Sox-2 and the Sox factors mentioned above. However, each of these Sox family members contains a serine-rich domain in its C-terminal region, which is necessary for transactivation (Figs. 1*B* and 3, *B* and *C*; Refs. 3, 22, and 23). Studies reported here using Gal4 fusion proteins illustrate that the Sox-2 region of amino acids 206–319 is able to transactivate when fused to the DNA-binding domain of Gal4. Although the region of amino acids 260–291 was not tested in these Gal4 constructs, it is unlikely that this region by itself can transactivate based on the results with the Sox-2 deletion, Sox-2$_{\Delta 207–254}$. This deletion construct includes amino acids 260–291 but lacks the serine-rich domain and exhibits significantly reduced transactivation, demonstrating the need for both the serine-rich domain and the C-terminal end for transactivation. Additional studies

using C-terminal deletions of Sox-2 demonstrate that the region of amino acids 121–319 is necessary for maximal synergistic transactivation in conjunction with Oct-3. However, considerable transactivation potential resides in the region containing the serine-rich domain and the C-terminal end (amino acids 206–319). These results further demonstrate the need for both the serine-rich domain and C-terminal end for transactivation.

The necessity of the last 28 amino acids for Sox-2 transactivation was surprising when compared with the results of experiments with fusion proteins of the Gal4 DBD and the chicken homolog of Sox-2, cSox-2. Sox-2 and cSox-2 are 93% identical within the last 28 amino acids. However, our Gal4/Sox-2 construct containing a deletion of the last 28 amino acids (Gal4/Sox-$2_{206–291}$) was unable to transactivate, whereas a Gal4/cSox-2 construct containing a deletion of the last 35 amino acids was reported to transactivate (24). This may be attributed to differences in the cell types utilized or to evolutionary differences between murine and chicken Sox-2.

Although there was a pronounced reduction in stimulation of transcription when the transactivation domain of Sox-2 was deleted, some synergistic transcriptional stimulation with Oct-3 and the truncated Sox-2 proteins was observed. Given that the DNA-binding domains of Sox-2 and Oct-3 have been shown to bind cooperatively with one another *in vitro* (4), it is likely that Sox-2 lacking the C-terminal transactivation domain can bind to the HMG motif and interact with Oct-3 to stabilize its binding to the neighboring POU motif. In this regard, stabilization of DNA binding by transcription factors has been observed for several other homeodomain proteins (41–44). Thus, cooperative binding of Sox-2 and Oct-3 to DNA may be partly responsible for the synergistic stimulation of transcription by these two transcription factors.

p300 Is a Candidate Co-activator for Sox-2 regulated Transcription

E1a is a viral protein known to influence the expression of many genes. E1a-like activity has been found in several cell types, but the endogenous protein(s) involved has yet to be identified. E1a has been shown to interact with several cellular proteins, including p300 and Rb, and to activate or repress the transcription of several genes (reviewed in ref. 26). For example, E1a inhibits *Rex-1* promoter-reporter gene constructs in EC cells (25). The *Rex-1* promoter contains two positive regulatory elements, a POU motif and a novel motif, necessary for *Rex-1* gene transcription in EC cells. Positive regulation of the *Rex-1* promoter requires binding of Oct-3 to the POU motif and binding of a protein designated Rox-1 to the novel (Rox) motif (25). Because the Rox and POU motifs are juxtaposed in a manner virtually identical to the HMG and POU motifs in the *FGF-4* enhancer and because in EC cells E1a reduces the expression of a *FGF-4* promoter-reporter construct containing the HMG and POU motifs,[4] we initially examined whether E1a could affect the stimulation of transcription mediated by Sox-2 and Oct-3. When expression vectors for E1a, Sox-2, and Oct-3 were co-transfected with the promoter-reporter gene construct pCatSO3, containing the HMG and POU sites, a dose-dependent inhibition of transcription was induced by E1a, even at extremely low concentrations of the E1a expression vector. These results suggest that E1a is inhibiting transcription either by directly interacting with Sox-2 and/or Oct-3 or by sequestering a co-activator that interacts with Sox-2 and/or Oct-3. It has been previously shown that E1a interacts directly with p300 (45, 46), and we determined here that a mutant

form of E1a that cannot bind p300 (mE1a) does not inhibit expression of pCatSO3 by Sox-2 and Oct-3. Therefore, our findings argue strongly that the effects of E1a are unlikely to be due to a direct interaction between E1a and Sox-2 and/or Oct-3. Instead, E1a is likely to be interacting with a co-activator of Sox-2 and/or Oct-3 to inhibit transcription.

Because mE1a does not inhibit Sox-2/Oct-3 stimulation of transcription, we examined a possible role for p300 in mediating the effects of Sox-2/Oct-3. p300 is a large nuclear protein found in most mammalian cells. It interacts with many different transcription factors (reviewed in ref. 47) and with the basal transcriptional machinery (48). However, p300 does not appear to bind directly to DNA. Therefore, p300 is thought to elicit its effects on transcription by acting as a bridging factor between the DNA-binding transcription factors and the basal transcriptional machinery. Overexpression of p300 has been shown to overcome the inhibitory effects of E1a on the *prolactin*, *p53*, *β-myosin heavy chain*, *HER-2/neu*, and *collagenase* promoters (27–31), suggesting that p300 plays a role in the transcription of these genes. Similarly, the work reported in this study argues that p300 is involved in the stimulation of transcription brought about by Sox-2/Oct-3. First, overexpression of p300 recovers the E1a inhibition of pCatSO3 transcription induced by Sox-2 and Oct-3. Second, in the absence of E1a, overexpression of p300 seemed to further stimulate the transcriptional synergism of Sox-2 and Oct-3. Third, and importantly, a p300 mutant (p300del30), which is unable to bind E1a, was also able to overcome the E1a inhibition of Sox-2/Oct-3 transcriptional activation. This last result argues that p300 is not sequestering E1a and preventing E1a from interacting with and inhibiting another co-factor required for Sox-2/Oct-3 activation. Instead, this result argues that E1a interacts directly with p300 and prevents p300 from stimulating transcription through Sox-2/Oct-3.

We also examined whether E1a affects the ability of Gal4/Sox-2 to transactivate. As observed with Sox-2/Oct-3 activation of pCatSO3, E1a, but not mE1a, inhibited Gal4/Sox-$2_{206–319}$ activation of the promoter-reporter construct pG5EC. Furthermore, p300 was able to rescue the E1a inhibition of Gal4/Sox-$2_{206–319}$ activation and further stimulate the transactivation of Gal4/Sox-$2_{206–319}$. Together, these studies provide strong evidence that p300 may be a co-activator of Sox-2/Oct-3 synergistic activation through the transactivation domain of Sox-2. In conclusion, the studies reported here describe several important findings. First, two different experimental promoters were used to map the location of the transactivation domain of Sox-2. Second, we determined that the co-activator p300 can mediate the effects of Sox-2. To our knowledge, this is the first report of a co-activator that mediates the action of a member of the Sox family of transcription factors, a family composed of at least 24 members. These findings raise several important questions that warrant further study, namely, does p300 directly interact with Sox-2? can p300 also mediate the effects of Oct-3? and does p300 mediate the effects of other members of the Sox family of transcription factors?

Acknowledgments – This work was supported by NCI, National Institutes of Health, Grant CA 74771. Core facilities of the University of Nebraska Medical Center Eppley Cancer Center used in the course of this work were supported in part by NCI Laboratory Cancer Research Center Support Grant CA 36727. Matthew Wiebe was supported in part by a fellowship provided by NCI-supported Training Grant CA 09476.

We thank Rob Lewis and Dave Kelly for critical reading of the manuscript. We thank Michael Wegner for plasmids Gal4/Sox-11 C3, Ivan Sadowski for plasmids pM3 and pG5EC, Joseph Nevins for plasmids CMV12S E1a and CMV12S E1aD2–36, and David Livingston for plasmids CMVp300 and CMVp300del30.

Notes

1. The abbreviations used are: HMG, high mobility group; EC, embryonal carcinoma; FGF, fibroblast growth factor; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; CMV, cytomegalovirus; ECF, enhanced chemifluorescence; gal, galactosidase; GFP, green fluorescent protein; DBD, DNA-binding domain; POU, pit, oct, and unc.
2. T. A. Luster and A. Rizzino, unpublished observations.
3. L. R. Johnson and A. Rizzino, unpublished observations.
4. C. Bernadt and A. Rizzino, unpublished data.

References

1. Wegner, M. (1999) *Nucleic Acids Res.* 27, 1409–1420.
2. Kuhlbrodt, K., Herbath, B., Sock, E., Hermans-Borgmeyer, I., and Wegner, M. (1998) *J. Neurosci.* 18, 237–250.
3. Kuhlbrodt, K., Herbarth, B., Sock, E., Enderich, J., Hermans-Borgmeyer, I., and Wegner, M. (1998) *J. Biol. Chem.* 273, 16050–16057.
4. Ambrosetti, D.-C., Basilico, C., and Dailey, L. (1997) *Mol. Cell. Biol.* 17, 6321–6329.
5. Ryan, A. K., and Rosenfeld, M. G. (1997) *Genes Dev.* 11, 1207–1225.
6. Pevny, L. H., and Lovell-Badge, R. (1997) *Curr. Opin. Genet. Dev.* 7, 338–344.
7. Nishimoto, M., Fukushima, A., Okuda, A., and Muramatsu, M. (1999) *Mol. Cell. Biol.* 19, 5453–5465.
8. Botquin, V., Hess, H., Fuhrmann, G., Anastassiadis, C., Gross, M. K., Vriend, G., and Scholer, H. R. (1998) *Genes Dev.* 12, 2073–2090.
9. Curatola, A. M., and Basilico, C. (1990) *Mol. Cell. Biol.* 10, 2475–2484.
10. Sasaki, A., Kubo, M., Hasan, S., Yano, Y., and Kakinuma, M. (1991) *Jpn. J. Cancer Res.* 82, 1191–1195.
11. Ma, Y.-G., Rosfjord, E., Huebert, C., Wilder, P., Tiesman, J., Kelly, D., and Rizzino, A. (1992) *Dev. Biol.* 154, 45–54.
12. Schoorlemmer, J., and Kruijer, W. (1991) *Mech. Dev.* 36, 75–86.
13. Dailey, L., Yuan, H., and Basilico, C. (1994) *Mol. Cell. Biol.* 14, 7758–7769.
14. Lamb, K., Rosfjord, E., Brigman, K., and Rizzino, A. (1996) *Mol. Reprod. Dev.* 44, 460–471.
15. Yuan, H., Corbi, N., Basilico, C., and Dailey, L. (1995) *Genes Dev.* 9, 2635–2645.
16. Johnson, L. R., Lamb, K. A., Gao, Q., Nowling, T. K., and Rizzino, A. (1998) *Mol. Reprod. Dev.* 50, 377–386.
17. Rosner, M. H., Vigano, M. A., Ozato, K., Timmons, P. M., Poirier, F., Rigby, P. W. J., and Staudt, L. M. (1990) *Nature* 345, 686–692.
18. Niswander, L., and Martin, G. R. (1992) *Development* 114, 755–768.

19. Yoshida, T., Muramatsu, H., Muramatsu, T., Sakamoto, H., Katoh, O., Sugimura, T., and Terada, M. (1988) *Biochem. Biophys. Res. Commun.* 157, 618–625.
20. Velcich, A., Delli-Bovi, P., Mansukhani, A., Ziff, E. B., and Basilico, C. (1989) *Oncogene Res.* 5, 31–37.
21. Tiesman, J., and Rizzino, A. (1989) *In Vitro Cell. Dev. Biol.* 25, 1193–1198.
22. van de Wetering, M., Oosterwegel, M., van Norren, K., and Clevers, H. (1993) *EMBO J.* 12, 3847–3854.
23. Ng, L.-J., Wheatley, S., Muscat, G. E. O., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P. L., Cheah, K. S. E., and Koopman, P. (1997) *Dev. Biol.* 183, 108–121.
24. Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R., and Kondoh, H. (1998) *Development* 125, 2521–2532.
25. Ben-Shushan, E., Thompson, J. R., Gudas, L. J., and Bergman, Y. (1998) *Mol. Cell. Biol.* 18, 1866–1878.
26. Zantema, A., and van der Eb, A. J. (1995) *Curr. Top. Microbiol. Immunol.* 199, 1–23.
27. Pfitzner, E., Jahne, R., Wissler, M., Stoecklin, E., and Groner, B. (1998) *Mol. Endocrinol.* 12, 1582–1593.
28. Sang, N., Avantaggiati, M. L., and Giordano, A. (1997) *J. Cell. Biochem.* 66, 277–285.
29. Hasegawa, K., Meyers, M. B., and Kitsis, R. N. (1997) *J. Biol. Chem.* 272, 20049–20054.
30. Chen, H., and Hung, M.-C. (1997) *J. Biol. Chem.* 272, 6101–6104.
31. Smits, P. H. M., de Wit, L., van der Eb, A. J., and Zantema, A. (1996) *Oncogene* 12, 1529–1535.
32. Scholtz, B., Lamb, K., Rosfjord, E., Kingsley, M., and Rizzino, A. (1996) *Dev. Biol.* 173, 420–427.
33. Kraus, V. B., Moran, E., and Nevins, J. R. (1992) *Mol. Cell. Biol.* 12, 4391–4399.
34. Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994) *Genes Dev.* 8, 869–884.
35. Sadowski, I., Bell, B., Broad, P., and Hollis, M. (1992) *Gene* 118, 137–141.
36. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* 264, 8222–8229.
37. Scholer, H. R., Ciesiolka, T., and Gruss, P. (1991) *Cell* 66, 291–304.
38. Imagawa, M., Miyamoto, A., Shirakawa, M., Hamada, H., and Muramatsu, M. (1991) *Nucleic Acids Res.* 19, 4503–4508.
39. Vigano, M. A., and Staudt, L. M. (1996) *Nucleic Acids Res.* 24, 2112–2118.
40. Brehm, A., Ohbo, K., and Scholer, H. (1997) *Mol. Cell. Biol.* 17, 154–162.
41. Hayashi, S., and Scott, P. (1990) *Cell* 63, 883–894.
42. van Dijk, M. A., and Murre, C. (1994) *Cell* 78, 617–624.
43. Chan, S. K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. S. (1994) *Cell* 78, 603–615.
44. van Dijk, M. A., Peltenburg, L. T., and Murre, C. (1995) *Mech. Dev.* 52, 99–108.
45. Egan, C., Jelsma, T. N., Howe, J. A., Bayley, S. T., Ferguson, B., and Branton, P. E. (1988) *Mol. Cell. Biol.* 8, 3955–3959.
46. Whyte, P., Williamson, N. M., and Harlow, E. (1989) *Cell* 56, 67–75.
47. Shikama, N., Lyon, J., and La Thangue, N. B. (1997) *Trends Cell Biol.* 17, 230–236.
48. Abraham, S. E., Lobo, S., Yaciuk, P., Wang, H.-G. H., and Moran, E. (1993) *Oncogene* 8, 1639–1647.