Transcription factor Sp3 antagonizes activation of the ornithine decarboxylase promoter by Sp1

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ABSTRACT

Ornithine decarboxylase (ODC) expression is important for proliferation and is elevated in many tumor cells. We previously showed that Sp1 is a major positive regulator of ODC transcription. In this paper we have investigated transcriptional regulation of rat ODC by the closely related factor Sp3. While over-expression of Sp1 caused a dramatic activation of the ODC promoter, over-expression of Sp3 caused little or no activation in either Drosophila SL2 cells (lacking endogenous Sp1 or Sp3) or in H35 rat hepatoma cells. Furthermore, co-transfection studies demonstrated that Sp3 abolished trans-activation of the ODC promoter by Sp1. DNase I footprint studies and electrophoretic mobility shift assays demonstrated that both recombiant Sp1 and Sp3 bind specifically to several sites within the ODC promoter also protected by nuclear extracts, including overlapping GC and CT motifs located between −116 and −104. This CT element is a site of negative ODC regulation. Mutation of either element reduced binding, but mutation of both sites was required to eliminate binding of either Sp1 or Sp3. These results demonstrate that ODC is positively regulated by Sp1 and negatively regulated by Sp3, suggesting that the ratio of these transcription factors may be an important determinant of ODC expression during development or transformation.

INTRODUCTION

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to putrescine, the first and normally rate limiting step in polyamine biosynthesis in mammalian cells (1–3). Putrescine is the precursor for the polyamines spermine and spermidine, which are necessary for cell growth and differentiation and have been implicated in the process of carcinogenesis (4). ODC enzymatic activity is induced transiently by various exogenous stimuli, including serum growth factors (5) and tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA; 6,7). Furthermore, ODC activity is critical for the G1/S transition during the cell cycle (1,2). Both the protein levels and enzymatic activity of ODC are elevated in cells transformed by various oncogenes, including v-src, neu and ras (8–10). The importance of ODC to neoplastic transformation is evident from the recent demonstration that over-expression of ODC is tumorigenic (11,12).

Consistent with its important role in cell growth, ODC is regulated at multiple levels, including transcription (13–15), translation (16) and enzyme stability (17). Mammalian ODC genes have been cloned from hamster (18), rat (19,20), mouse (21) and human (22) and are highly conserved, both within the coding region and presumptive transcription regulatory sequences. Among these species, the 5′-flanking region has 82% identity within the first 148 bp, with reduced conservation seen over the next 380 bp. The 5′-flanking region of the rat ODC promoter contains numerous potential transcription factor binding sites, including Sp1, AP2, CRE, a CCAAT-like motif and an ETS motif (23).

Although the functional significance of these binding sites is not well understood, some progress has been made in determining their relative importance (23). The CCAAT-like motif (CCgAT, –84 to –80) appears to be important for basal expression of ODC in Rat-1 fibroblasts (20). A GC-rich region (−152 to −107), together with a conserved CRE (−50 to −42), was shown to contribute to both basal and protein kinase A-induced ODC transcriptional activity (24). A novel CRE binding protein that mediates the cAMP responsiveness of the mouse ODC promoter has been reported (25). Our laboratory recently confirmed the importance of the CRE site for basal expression of ODC in both rat H35 hepatoma cells and Rat-2 fibroblasts (26) and demonstrated that both TPA and serum induction of ODC are mediated in a cell type-specific manner through sequences between −92 and +13. This region lacks conventional AP-1 binding sites or serum response elements (26). However, Wrighton and Busslinger recently reported that ODC transcription may be directly regulated by c-fos (27), suggesting a pathway for ODC induction in response to extracellular signals.

Both the murine and human ODC genes are also trans-activated by c-Myc (28–30). In the murine ODC gene, c-Myc was shown to bind to two conserved sequences in the first intron and to regulate expression of reporter plasmids containing these sequences. In contrast, the human ODC gene was reported to be regulated by c-Myc–Max heterodimers through a 5′-flanking element that is not conserved in mouse, rat or hamster ODC genes (29). However, ODC promoter fragments lacking either of these putative Myc regulatory elements remain fully inducible by serum (26) and TPA (26,31).

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Several recent reports have highlighted the potential importance of Sp1 in the regulation of ODC promoter activity (32–34). We demonstrated that a GC-rich region (~342 to −93) containing five consensus Sp1 binding sites (GGGCCG, GC boxes I–V), which is critical for ODC expression in mammalian cells, binds Sp1 in vitro and that Sp1 dramatically trans-activates ODC in Drosophila SL2 cells (32). Asad et al. (34) confirmed that ODC promoter activity was correlated with the number of intact GC boxes and their affinity for Sp1 protein. Of particular interest, cell type-specific regulation of ODC promoter activity was reported to involve Sp1 and an uncharacterized negative factor, referred to as NF-ODC1 (33), binding to overlapping sequences between −118 and −100 in a mutually exclusive manner. This sequence is highly conserved in mammalian ODC genes and is clearly protected in DNase I footprinting assays (32). This region (~118 to −100) includes GC box V, as well as a CCCTCCC motif (CT element). In the c-myc promoter, Sp1 has been shown to bind to similar CT elements (35). The reported NF-ODC1 binding site in the ODC promoter includes the CT element, suggesting that NF-ODC1 might antagonize Sp1.

In this regard, three sequences encoding proteins (Sp2, Sp3 and Sp4) which are closely related to transcription factor Sp1 have been recently cloned (36,37). Together, these proteins make up the Sp1 multigene family. The DNA binding domains of Sp1, Sp3 and Sp4 are highly conserved and bind the GC-rich sequence with identical affinities; they also bind CT elements with comparable, albeit slightly weaker, affinity. In contrast, Sp2 appears to have distinct DNA binding specificity (37). Like Sp1, Sp3 is ubiquitous. However, various reports differ on whether Sp3 is a transcriptional activator (38,39) or inhibitor (40–43), indicating that the function of Sp3 may be context- or cell type-dependent. Thus, we felt it was important to investigate the role of Sp3 in ODC regulation.

Here we report regulation of the rat ODC promoter by Sp3. To determine the functional effects of Sp3 on ODC transcription, we co-transfected Sp3 expression vectors, in the presence or absence of Sp1 expression vectors, with ODC reporter genes. While Sp1 is a strong activator of the ODC promoter, Sp3 failed to activate the ODC promoter in either Drosophila Schneider SL2 cells or H35 rat hepatoma cells. In addition, enforced expression of Sp3 repressed Sp1-mediated trans-activation in both cell types. Electrophoretic mobility shift assays (EMSA) and DNase I footprinting analysis demonstrated that both recombinant Sp1 and Sp3 bind specifically to the ODC promoter and that they protect regions of the promoter also protected by H35 nuclear proteins. Furthermore, using specific antibodies against either Sp1 or Sp3 showed that both proteins bind to the ODC promoter in H35 nuclear extracts. These results strongly suggest that changes in the cellular ratio of Sp1 and Sp3 activity could dramatically affect ODC expression.

**MATERIALS AND METHODS**

**Materials**

Enzymes were purchased from Boehringer Mannheim (Indianapolis, IN) and Promega Corp. (Madison, WI). Radiomucleotides were obtained from DuPont NEN (Wilmington, DE). Recombinant transcription factor Sp1 was purchased from Promega. Rabbit antiserum directed against human Sp1, Sp3 and Sp4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Double-stranded synthetic oligonucleotides containing consensus transcription factor binding sites were purchased from Promega or Santa Cruz Biotechnology and mutant oligonucleotides were obtained from Santa Cruz Biotechnology or were custom synthesized by Genosys (Houston, TX). Coomassie reagent for protein determination was from Pierce Chemicals (Rockford, IL). All other chemicals were from Sigma Chemicals (St Louis, MO).

**Plasmids**

Details of the ODC reporter plasmids used in this study have been published elsewhere (26,32). The expression vectors pPac-control and pPac-Sp1 were a kind gift of Dr Robert Tjian (University of California, Berkeley, CA). The expression vectors pPac-Sp3, pCMV-Sp3 and pGST-Sp3 were gifts from Dr Guntram Suske (Institut für Molekularbiologie und Tumorforschung, Marburg, Germany). The parental GST expression vector, pGEX2T, was obtained from Dr Susan M. Fischer (University of Texas M.D. Anderson Cancer Center, Smithville, TX) and pCMV-Sp1 was obtained from Dr Sophia Y. Tsai (Baylor College of Medicine, Houston, TX).

**Transfections and luciferase assay**

Subconfluent Reuber H35 rat hepatoma cells were transfected by electroporation with 10 µg/dish ODC reporter plasmid (pOD-Clux2m) and 3.3 µg/dish pCMV-Sp1 or pCMV-Sp3 plus 1.6 µg/dish pCMV-β-gal. The total amount of transfected DNA was kept constant by adding control plasmid (pcDNA-3). Cells were harvested 48 h after transfection and were lysed in 100 µl lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100 and 1 mM DTT). Extracts were clarified by centrifugation and the protein concentration was determined by the Bradford method (44). Luciferase and β-galactosidase activities were assayed as described below.

*Drosophila melanogaster* Schneider SL2 cells were obtained from the American Type Culture Collection and grown at 27°C in Schneider’s medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (45). The cells were transfected as described using the calcium phosphate precipitation method (46,47). Cells were seeded at a density of ~4×10^4 in a 60 mm dish 24 h prior to transfection with 5 µg luciferase reporter plasmid and varying amounts of pPac-control, pPacSp1 or pPacSp3 expression plasmids, as indicated in Results. The cells were harvested 48 h later, washed thrice with cold phosphate-buffered saline (PBS), lysed in 500 µl lysis buffer and assayed as described below.

Luciferase activity was assayed in aliquots containing equal amounts of protein with reagents obtained from Promega. β-Galactosidase activity was determined using a chemiluminescent substrate according to the manufacturer’s recommendations (Tropix, Bedford, MA). Both assays were linear throughout the range of results reported. Results were expressed as the ratio of luciferase to β-galactosidase activity at equal amounts of protein for mammalian cells. Results from SL2 cell transfections were expressed as luciferase activity (equal protein concentration) normalized to that obtained with an empty expression vector, since expression of β-galactosidase activity from either pCMV-β-Gal or pRSV-β-Gal was insufficiently reliable for accurate determination in SL2 cells. Each experiment represents the mean of three dishes of cells and was repeated at least three times, using two or more independent plasmid preparations.
Preparation of nuclear and protein extracts

Reuber H35 rat hepatoma cells were maintained as previously described (48). The H35 cells were grown to subconfluence and serum starved for 60–72 h. Nuclear extracts were made essentially by the method of Dignam et al. (49) with minor modifications as described (32). Protein content of the nuclear extracts was determined by the method of Bradford (44).

Purification of GST fusion proteins

Cultures of Escherichia coli DH5α transformed with pGEX-2T or pGST-Sp3 were grown to an absorbance at 600 nm of 0.8–0.9 and fusion protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactosidase (IPTG). Cells were harvested after 3 h induction, washed thrice with PBS and resuspended in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin and leupeptin and 5 μg/ml pepstatin and sonicated for 1 min. After 30 min incubation at room temperature, the cell debris was removed by centrifuging at 12 000 g for 15 min. The supernatant was mixed with glutathione–Sepharose beads pre-equilibrated with PBS and incubated at room temperature for 1 h. The beads were washed with PBS thrice and the bound proteins were eluted with glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0). Western blot analysis of the eluted protein was performed using an antibody against Sp3 detected a single prominent band corresponding to the expected molecular weight of 120 kDa for the fusion protein. Extracts from E.coli transformed with the parental pGEX-2T plasmid did not cross-react with anti-Sp3.

Protein–DNA binding studies

EMSA, competition analysis and antibody supershifts were carried out as described (32). Plasmid pODC 250 was digested with EcoRI and HindIII and the ODC promoter fragment (~345 to ~93) was isolated by electroelution according to standard methods (50). This fragment was labeled with [α-32P]dCTP (3000 Ci/mmol) using the Klenow fragment of DNA polymerase I and was purified by phenol/chloroform extractions followed by ethanol precipitation. ApaI digestion was used to isolate the ~168 to ~93 fragment. The method of Galas and Schmitz (51) was used for DNase I footprinting as described (32).

The Southwestern blotting technique was adopted from Cowell and Hurst (53). Briefly, protein extracts were resolved by 10% SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane. After transfer, the membrane was washed in 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA and 1 mM DTT (wash buffer) and blocked overnight with 2.5% (w/v) non-fat dried milk in Tris–HCl (wash buffer) and blocked overnight with 2.5% (w/v) non-fat dried milk in Tris–HCl, pH 8.0, 1 mM MgCl2, 10% (v/v) glycerol, 50 mM NaCl and 1 mM EDTA, followed by incubation with the DNA probe and poly(dI·dC) as a non-specific competitor for 4 h. After this, the filter was washed with wash buffer, dried and autoradiographed.

Western blot analysis

Nuclear proteins were fractionated on 10% SDS–polyacrylamide gels (52) and were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (blocking solution). They were next incubated with primary antibody against Sp3, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham, IL) in blocking solution. Bound antibody was detected by enhanced chemiluminescence, following the manufacturer’s directions (Amersham Corp).

RESULTS

Sp3-mediated repression of ODC trans-activation by Sp1

We previously demonstrated that sequences between –409 and –93 of the rat ODC promoter, containing five consensuses GC boxes, are stimulated several hundred-fold by co-transfected Sp1 in Drosophila SL2 cells (32). SL2 cells were used for these transient expression studies, since they do not express endogenous Sp1 or other members of the Sp1 family (40–43,47,54–55). To determine the effect of Sp3 on ODC promoter activity, we co-transfected SL2 cells with pODClux2m (containing ODC sequences –409 to +13 fused to the firefly luciferase coding sequence) and expression vectors for either Sp1 or Sp3, in which expression is under control of the Drosophila actin promoter. As we previously reported (32), pPacSp1 enhances expression dramatically (Fig. 1, inset). Indeed, co-transfection of high concentrations of pPacSp3 occasionally inhibited the activity of pODClux2m even below the low basal level (data not shown). Thus Sp1, but not Sp3, efficiently activates the ODC promoter in SL2 cells. This result prompted us to determine the effect of Sp3 on Sp1 trans-activation of the ODC promoter. SL2 cells were co-transfected with pODClux2m and 0.5 μg pPacSp1 alone or together with increasing amounts of pPacSp3. The total amount of DNA added was kept constant using the parental pPac plasmid. We used a higher ratio of pPacSp3 to pPacSp1, since EMSA data with a consensus GC box probe indicated that DNA binding activity due to the trans-activated pPacSp3 was ∼25% of that seen with a comparable amount of pPacSp1 (data not shown). The addition of pPacSp3 in the presence of the Sp1 expression plasmid led to a profound inhibition of >90% of Sp1-stimulated ODC promoter activity (Fig. 1, bars). Thus, not only does Sp3 fail to trans-activate the ODC promoter in SL2 cells, it also represses the potent effect of Sp1.

Effect of Sp1 and Sp3 on ODC promoter activity in mammalian cells

In order to determine the effect of Sp1 family members on ODC promoter activity in mammalian cells, similar co-transfection experiments were performed in H35 cells. For this experiment, the pODC168m reporter (containing ODC sequences from –168 to +13) was used, since this proximal portion of the promoter is most highly conserved among mammalian ODC genes. This reporter contains GC box V, but lacks GC boxes I–IV present in pODClux2m. Over-expression of Sp1 using 3.3 μg/dish pCMV-Sp1 in H35 cells activated the ODC promoter by ∼3.4-fold (Fig. 2, hatched bar). In contrast, co-transfection of 3.3 μg/dish pCMV-Sp3 had virtually no effect on ODC promoter activity (stippled bar). Furthermore, co-expression of equal concentrations of pCMV-Sp1 and Sp3 together with Sp1 abolished trans-activation of the ODC promoter by Sp1 (cross-hatched bar). Because of the high background of endogenous Sp1 and Sp3 (see below), it was not feasible to determine the amount of exogenous Sp1 or Sp3.
while lane 2 is the result obtained in the presence of extracts from (Fig. 3). Lane 1 is the DNase I digest obtained with free DNA, by performing DNase I footprinting experiments using GST–Sp3 have been implicated as binding sites for Sp1 family proteins. These elements includes GC box V (32). In the present study we have used EMSA footprinting that nuclear proteins of H35 cells bind regions of the ODC promoter designated PR-I and PR-II (–93 to –120 and –304 to –332 respectively). PR-II includes GC boxes I–IV, while PR-I includes GC box V (32). In the present study we demonstrated by EMSA and DNase results were obtained in Rat-2 fibroblasts and COS-7 cells over a range of concentrations of transfected pCMV-Sp1 or pCMV-Sp3 or when using pODClux2m as a reporter (data not shown). Taken together, these results indicate that Sp3 specifically inhibits Sp1 trans-activation of ODC promoter activity in mammalian cells, as well as in SL2 cells.

Binding of recombinant Sp1 and Sp3 to the ODC promoter

In a previous publication we demonstrated by EMSA and DNase I footprinting that nuclear proteins of H35 cells bind regions of the ODC promoter designated PR-I and PR-II (–93 to –120 and –304 to –332 respectively). PR-II includes GC boxes I–IV, while PR-I includes GC box V (32). In the present study we have used EMSA to demonstrate that recombinant Sp1 and GST–Sp3 were also able to bind ODC probes containing PR-II (ODC probe –345 to –168) and PR-I (ODC probe –168 to –93). The binding was highly specific, as it was not reduced by competition with a 100-fold molar excess of non-specific oligonucleotides, including an AP-1 consensus or a mutant GC box consensus binding site (data not shown). To obtain more precise information on the binding of Sp1 and Sp3 to the ODC promoter, we used DNase I footprinting. Further inspection of the ODC sequence revealed that PR-I contains two potential CT elements (–110 to –104 and –98 to –93), one of which partially overlaps GC box V. These elements are highly conserved in mammalian ODC genes and CT elements have been implicated as binding sites for Sp1 family proteins. Therefore, we investigated potential binding of Sp3 to this region by performing DNase I footprinting experiments using GST–Sp3 (Fig. 3). Lane 1 is the DNase I digest obtained with free DNA, while lane 2 is the result obtained in the presence of extracts from pGE-2T-transformed bacteria. Lane 3 shows protection by nuclear extracts from H35 cells, which extends from –130 to –93, as previously reported (32). GST–Sp3 protected 100 to –120, which includes both GC box V and CT element I, but not CT element II (lane 4). This result suggested that Sp3, as well as Sp1, could contribute to the PR-I protection pattern obtained with nuclear extracts.

Mutational analysis

Based on the DNase I footprint result, it appeared that both Sp1 and Sp3 may bind not only GC box V, but also the overlapping CT element I motif (–110 to –104). Therefore, we synthesized double-stranded oligonucleotides containing the sequence –93 to –124 and the same oligonucleotide with a mutation in either GC box V, CT element I or both (see Materials and Methods). The wild-type sequence was used as an EMSA probe with recombinant Sp1 or GST–Sp3 or crude H35 nuclear extracts (Fig. 4A and B). Two complexes were formed with GST–Sp3, but only one major complex was observed with either recombinant Sp1 or with H35 extract. No binding was observed with extracts prepared from bacteria transformed with the empty pGEX-2T plasmid (data not shown). The specificity of these complexes was confirmed by competition analysis using homologous and heterologous double-stranded synthetic oligonucleotides. Competition studies were performed with unlabeled GC, CT or GCCT mutants (Fig. 4). The wild-type ODC –124/–93 competitor completely abolished binding. At a 100-fold molar excess, the GC box V mutant competitor reduced binding of GST–Sp3 or H35 extracts by 70–80%; inhibition of Sp1 binding was reproducibly 60%. The CT element I mutant competitor had a similar effect in each case. The double mutant GC-CT competitor failed to compete for either Sp1 or H35 nuclear extract binding, but was capable of marginal competition for GST–Sp3 binding. Additional EMSA experiments were performed using the GC box V, CT element I or GC-CT mutant oligonucleotides as probes (data not shown). With the single mutants, the binding of H35 extract or GST–Sp3 was dramatically reduced, while the binding of Sp1 was reduced...
Figure 3. DNase I footprinting analysis of PR-I. A single end-labeled ODC promoter fragment (–168 to –93) was incubated for 25 min at room temperature with protein extracts. The DNA–protein complexes were treated with DNase I for 2 min, following which the DNA was purified and resolved on 8% sequencing gels. Incubations were as follows: lane 1, no nuclear extract (free DNA); lane 2, extract from bacteria transformed with parental pGEX-2T; lane 3, nuclear extract from H35 cells; lane 4, recombinant GST–Sp3 (20 µg). The region protected by nuclear extracts (PR-I) is shown schematically by a white box. The positions of GC box V (hatched ovals) and CT motifs I and II (cross-hatched ovals) are also indicated (see text for discussion).

Identification of Sp1 and Sp3 in the DNA–protein complexes

We previously showed, based on Western blot analysis and supershift studies, that H35 nuclear extracts contain Sp1 and that Sp1 interacts specifically with both ODC –345 to –168 and –168 to –93 (32). In the present study we have examined expression of Sp3 in H35 cells (Fig. 5A, lane 1). The Sp3 antibody detected a band of ∼97 kDa, a doublet at 65–70 kDa and a non-specific band of 44 kDa; these results are similar to the pattern previously reported from HeLa cells (40). The specificity of these bands was demonstrated using the blocking peptides for anti-Sp3 antibody (lane 2). In contrast, we previously found that antibody against Sp1 detected a protein doublet of ∼95 kDa in H35 nuclear extracts (32).

To determine if the Sp3 present in H35 nuclear extracts binds the ODC promoter, we performed supershift experiments (Fig. 5B). Preincubation of nuclear extracts with Sp1 antibody followed by EMSA using the –168 to –93 probe caused supershift of a significant part of complex I, indicating the presence of Sp1 in this complex, as previously reported (32). When we used Sp3 antibody in EMSA, the faster migrating complex II was eliminated, along with an apparent supershift of a portion of complex I. This result demonstrated that H35 nuclear extracts contain proteins antigenically related to both Sp1 and Sp3 and that both interact with the –168 to –93 ODC promoter element. As a negative control, anti-c-Jun, normal rabbit serum or the blocking peptide for Sp1 or Sp3 were included in EMSA experiments. None of these affected the mobility of the complexes (Fig. 5B and data not shown).

The in vitro binding studies and footprinting analysis suggested that multiple proteins, including Sp1 and Sp3, interact in a sequence-specific manner with ODC promoter sequences between –345 and –93. Southwestern analysis was done to determine the molecular weights of these proteins. H35 hepatoma nuclear and cytoplasmic extracts were separated by SDS–PAGE and probed with the radiolabeled –345 to –93 ODC promoter fragment. As shown in Figure 5C, at least seven different bands were consistently detected. The two most intense of these, with approximate molecular weights of 95–100 kDa, migrated at approximately the positions of full-length Sp1 and Sp3. Additional bands of 60–65 and 30–35 kDa were also consistently observed. No DNA binding proteins were detected in cytoplasmic extracts.
When both Sp1 and Sp3 were co-expressed in SL2 cells, Sp3 abolished trans-activation of the ODC promoter by Sp1 (Fig. 1). Similarly, Sp3 failed to trans-activate the ODC promoter in H35 cells, while Sp1 caused a 3- to 4-fold increase in ODC promoter activity (Fig. 2). Strikingly, co-transfection of Sp3 along with Sp1 completely abolished the stimulatory effects of Sp1 on the ODC promoter in H35 cells (Fig. 2). We were unable to detect the presence of Sp4 in H35 cells by Western blot analysis or by supershift EMSA (data not shown). Although interpretation of the mammalian transfections is complicated by the abundance of both Sp3 and Sp1 (Fig. 5; 32), the results were consistent with Sp3 inhibition of Sp1 trans-activation, as observed in the SL2 cells.

Both recombinant Sp1 and Sp3 bind to the −345 to −168 probe, containing four consensus GC boxes, and to the −168 to −93 probe, containing GC box V and putative CT elements (data not shown). DNase I footprinting experiments demonstrated that PR-I of the ODC promoter includes potential overlapping binding sites from −116 to −104 (5′-CCCCGCCCTCCCCC-3′) for Sp1 and Sp3. This region is fully conserved in human, mouse and rat ODC promoters, suggesting a functional significance (19;20). The 5′-end of the sequence contains a consensus GC box and the 3′-sequence includes a CT element; each of these motifs is known to bind both Sp1 and Sp3 (35;42). In this regard, sequences containing this CT element have been reported to mediate negative regulation of ODC by an uncharacterized transcription factor referred to as NF-ODC1 (53). Both recombinant Sp3 and nuclear extracts protected this sequence against DNase I digestion (Fig. 3). Because of its negative effects on ODC transcription, it is formally possible that Sp3 may be equivalent to the uncharacterized negative factor NF-ODC1, although this remains to be confirmed. Underscoring the potential importance of this region to ODC regulation is the recent report that WT1 also binds to sequences with overlapping GC box V and the CT elements (60).

Our experiments further demonstrate that proteins immunologically related to both Sp1 and Sp3 comprise the major protein–DNA complexes formed with H35 nuclear extracts and the ODC −168 to −93 probe (Fig. 5B). Western blot analysis of nuclear extracts using anti-Sp3 identified a band migrating with a molecular weight of ∼97 kDa and a doublet of 69 kDa (Fig. 5A), consistent with previous findings in HeLa nuclear extracts (40). Also, in vitro translated Sp3 mRNA was reported to produce products of ∼100 and 69 kDa (37). Although the relationship between these bands is not clear, it is possible that the smaller forms may result from differential utilization of translation start signals (37). It is also possible that they arise through alternative splicing, as a 48 kDa form of Sp1 produced by alternative splicing has been reported (61).

Southwestern analysis (Fig. 5C) confirmed that the proteins binding to the −345 to −93 ODC probe included species of ∼95–97 and 69 kDa, as well as an additional protein migrating at 30–35 kDa. Neither anti-Sp1 nor anti-Sp3 recognized a 30–35 kDa protein in Western blots. However, this 30–35 kDa species may also be a zinc finger protein, since the zinc chelator o-phenanthroline eliminated all protein–DNA complexes observed by EMSA of the −345 to −93 probe with H35 proteins, while ZnCl2 restored them (data not shown). Gel supershift studies also indicated a faster migrating band not affected by anti-Sp1 or anti-Sp3 or by both antibodies together (Fig. 5B and data not shown). Taken together with the transfection results (Figs 1 and 2), these in vitro
studies clearly demonstrate that Sp1 and Sp3 are critical determinants of ODC transcription. The consensus Sp1 recognition sequence, or GC box, has been given as either the hexanucleotide GGCCGG or the decanucleotide (GT)GGCCGG(G/A)(G/A)(C/T) (62). However, Sp1 binds a variety of related sequences with nearly equal affinity. In particular, substitution of A or T for the underlined C in the consensus sequence results in no more than a 6-fold loss of affinity (63). Based on the sequence similarity of the DNA binding domains of Sp1 and Sp3, it is likely that both proteins can recognize similar binding sites, and this has in fact been demonstrated for binding to both GC and GT motifs (36). Thus, the observed negative effect of Sp3 on ODC promoter activity may be due to direct competition between Sp3 and Sp1 for binding sites, thereby inhibiting Sp1-dependent trans-activation of the ODC promoter. However, these differential effects of Sp1 versus Sp3 may be dependent on promoter context or other factors, since Sp3 is a positive regulatory factor in the SIS/PDGF-β gene (39). Furthermore, Udvadia et al. (38) reported that both Sp1 and Sp3 trans-activate the c-fos, c-myc and TGF-β promoters through a ‘retino blastoma control element’ common to these promoters and this effect was further activated by co-expression of the retino blastoma protein (64).

In addition to competition for DNA binding sites, direct repression, quenching, squelching and steric hindrance are mechanisms that have been suggested to explain negative interference of transcription factors in gene expression (65,66). Based on the present data, we propose that Sp3 repression of Sp1 trans-activation of the ODC promoter involves competitive DNA binding. This conclusion is consistent with published reports of the effects of Sp1 and Sp3 on the uroteroglobin, HIV-I and c-myc promoters (40–42). However, the possibility of squelching or steric hindrance as a repression mechanism cannot be ruled out. The glutamine-rich activation domain of Sp1 interacts with dTAFII110 and TBP (67). While this manuscript was in preparation, it was reported that the homologous domain of Sp3 is also a functional activation domain (68). However, Sp3 also contains a repressor activity that has been mapped to a 72 amino acid region at the 5’-end of the zinc finger DNA binding region (69); repression by this domain appears to be highly dependent on binding site context and cell type. Thus, availability of specific co-activators, co-repressors or other transcription factors may determine whether Sp3 activates or inhibits transcription of a specific gene.

In conclusion, this study demonstrates that the transcription factor Sp3 inhibits Sp1-mediated trans-activation of rat ODC promoter activity. Thus differences in the ratio between Sp1 and Sp3 could be critical for the aberrant expression of ODC promoter activity during carcinogenesis or in other physiological contexts. In this regard, it would be interesting to compare the expression of Sp1 and Sp3 between normal cells and transformed cells showing elevated levels of ODC activity.

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REFERENCES