Functional Interactions between the Estrogen Receptor and the Transcription Activator Sp1 Regulate the Estrogen-dependent Transcriptional Activity of the Vitellogenin A1 io Promoter*

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Two distinct, TATA box-containing promoters regulate the transcriptional activity of the Xenopus vitellogenin A1 gene. These two promoters are of different strength and are separated by 1.8 kilobase pairs of untranslated sequence. Estrogen receptor (ER) and its ligand, 17β-estradiol, induce the activity of both promoters. The estrogen response elements (EREs) are located proximal to the downstream io promoter while no ERE-like sequences have been identified in the vicinity of the upstream io promoter. We show here, that transcriptional activity of the upstream io promoter is Sp1-dependent. Moreover, we demonstrate that estrogen inducibility of the io promoter results from functional interactions between the io bound Sp1 and the ER bound at the proximity of i. Functional interactions between Sp1 and ER do not require the presence of a TATA box for transcriptional activation, as is demonstrated using the acyl-CoA oxidase promoter. The relative positions that ER and Sp1 occupy with respect to the initiation site determines whether these two transcription activators can synergize for transcription initiation.

Transcription is one of the levels at which eukaryotic gene expression is controlled. Regulation at this level is achieved through the coordinate and cooperative action of regulatory proteins collectively known as transcription factors. Proteins that regulate RNA polymerase II-dependent gene expression include the factors that associate with RNA polymerase II and form the transcription initiation complex as well as activators that are not directly involved with the recruitment of polymerase II to the promoter (reviewed in Refs. 1 and 2). These latter proteins bind specific DNA elements located either upstream or downstream of the core promoter and modulate basal and signal-dependent transcription in a ubiquitous or specific manner. Nuclear hormone receptors (NHR)1 form the best characterized class of transcription factors that are activated by extracellular signals (reviewed in Refs. 3–5). Ligand binding to NHR is correlated with the transcriptional activation or inhibition of target genes. In some instances it is a prerequisite for the entry of the receptor into the nucleus or for DNA binding (reviewed in Ref. 6). Binding of NHR to target promoters requires the presence of a hormone response element. These elements, for the most part, consist of two copies of a hexanucleotide core sequence, which can have direct repeat, palindrome, or inverted palindrome configurations (reviewed in Ref. 7).

Contrary to the NHRs that control gene expression in developmental stage-, tissue-, sex specific- and ligand-dependent manner, ubiquitous transcription activators are responsible for the basal expression level of a large number of genes. One such prototypic transcription factor is Sp1, a ubiquitous eukaryote protein that activates transcription through its glutamine-rich transactivation domain (8). As is the case for the nuclear receptors, Sp1 binding to DNA and subsequent activation of target genes requires the presence of specific regulatory sequences on the promoters of these genes, known as GC boxes with the consensus sequence 5′-GGCGGG-3′ (9).

The action of transcription activators is thought to be mediated through direct interactions of the activation domain(s) of these proteins with components of the basal transcription machinery (10). For instance, Sp1 has been shown to interact with the TAF1110 component of the TFIID complex (11), while members of the NHR superfamily, such as the estrogen receptor and COUP-TFI, have been reported to interact with TAF1130 and TFIIB, respectively (12, 13). Such interactions could be responsible for the recruitment of additional basal transcription complex factors, the stabilization of the transcription complex and possibly promoter chromatin rearrangements, which result in an increased transcriptional activity from the target promoter.

Promoters of genes regulated by NHRs can contain, in addition to the hormone response element, binding sites for other transcriptional activators; an additional mechanism of transcriptional fine tuning is, thus, possible through functional interactions between distinct transcription factors. Indeed, we have shown previously that the estrogen receptor can transcriptionally synergize with the proline-rich transactivation domain of CTF/NF-1 on synthetic promoters (14) and that the peroxisome proliferator-activated receptor (PPAR/retinoid X receptor (RXR) heterodimer synergizes with Sp1 for the activation of the rat acyl-CoA oxidase (ACO) promoter (15).

The genes coding for the egg yolk proteins vitellogenins (vtg) are among the few known targets of direct estrogen receptor action (16). In Xenopus laevis four genes, A1, A2, B1 and B2, terized class of transcription factors that are activated by extracellular signals (reviewed in Refs. 3–5). Ligand binding to NHR is correlated with the transcriptional activation or inhibition of target genes. In some instances it is a prerequisite for the entry of the receptor into the nucleus or for DNA binding (reviewed in Ref. 6). Binding of NHR to target promoters requires the presence of a hormone response element. These elements, for the most part, consist of two copies of a hexanucleotide core sequence, which can have direct repeat, palindrome, or inverted palindrome configurations (reviewed in Ref. 7).

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have been identified that encode distinct but closely related vtgs. All four genes are expressed in developmental stage (adult), tissue- (hepatocyte), sex- (female), and hormone (estrogen)-dependent manner (reviewed in Ref. 17). The promoter of the B1 gene is the best characterized in terms of identification of transcription factor binding sites and nucleosome positioning (18, 19). Thus, in addition to the TATA box and estrogen-responsive unit (ERU), there exist several CTF/NF-I, C/EBP, and HNF-3 binding sites. The vtg A1 gene promoter, which has not been studied extensively so far, has a unique feature that consists of the presence of a functional transcription initiation site, io, located 1.8 kbp upstream of the major transcription initiation site, i (20). In this work, we have studied the mechanism of estrogen-dependent stimulation of the upstream io promoter. We demonstrate that transcriptional activity from io is mediated by Sp1, which by functional interactions with the estrogen receptor, bound proximal to the io transcriptional initiation site, confers estradiol inducibility to this promoter.

MATERIALS AND METHODS

Construction of Recombinant Plasmids—The reporter plasmids pA1-CAT (1899/6), pA1- (1995/6)-CAT, pio-CAT.ERE, and pio.CAT were described previously (20). The ERE.ACO.I1-LUC contains a single copy of the vtg A2 ERE cloned upstream of position –208 of the rat acyl CoA-oxidase promoter (21). It was constructed by cloning the ERE.ACO–208/-20 BglII-HindIII fragment in the corresponding sites of the pG-L2 basic vector (Promega). Vectors pio.TATA.i1 and pio.GC.i1 were constructed by cloning PCR amplified fragments containing sequences from –1976 to –1803 and from –1976 to –1848, respectively, of the A1 promoter at position –580 (Pst1 site) of the pA1–580/+6)-CAT plasmid. The Xenopus estrogen receptor cloned in the expression plasmid pSG5 (pSG5.xER) has been described previously (22). The expression vector for Sp1 (pPADD.Sp1) has also been described previously (23).

The recombinant plasmid Sp1-24 contains the three GC boxes of the io promoter, included in the 63-bp HpaII-HhaI fragment spanning positions from –1900 to –1837 of the vtg A1 promoter, in the Smal site of the pGEM3 vector (Promega). The plasmid Sp1-24 was constructed by cloning the 62-bp Ncol-FokI fragment, from positions 107 to 168 of the pSV2CAT plasmid (23), which contains the five GC boxes of the SV40 early promoter, into the Smal site of pGEM3. Plasmid E1V40 contains a 1900-bp fragment of positions 180–3900 of the SV40 early promoter and is methylated with dimethyl sulfate as described in Ref. 28. The chemically modified probes were used in EMSA in the conditions described above but scaled up five times. Protein-DNA complexes as well as the free probe, electrophoretically separated and visualized by autoradiography, were excised from the gel by electroelution for 2 h at 4 °C in TAE buffer (0.08 M Tris acetate, 0.002 M EDTA). Following electroelution, an equal volume of a solution containing 50 µg of proteinase K, 20 mM EDTA, and 1% SDS was added to the samples, which were then incubated for 30 min at 45 °C. The proteinase K digestion was followed by phenol/chloroform extractions before the DNA was precipitated and cleaved with 1 µl piperidine for 30 min at 95 °C. The cleaved fragments were washed several times with 50% methanol before electrophoresis on a 12% polyacrylamide, 7.5 M urea gel.

In Vitro Transcription—Transcription reactions were performed in a buffer containing 2.6 mM Tris-HCl, pH 8.0, 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM MgCl2, 0.5 mM NpSO4, 0.13 mM ZnCl2, 0.5 mM EDTA, 0.015 mM EGTA, 0.5 mM dithiothreitol, 2.6 mM Tris-acetate, pH 8.0, 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM MgCl2, 0.5 mM MnSO4, 0.13 mM ZnCl2, 0.5 mM EDTA, 0.015 mM EGTA, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, 4 mM spermidine, 0.5 mM creatine phosphate, 0.6 mM of each of ATP, CTP, GTP, and UTP, 40 units of RNasin (Promega), 1 µg of sonicated salmon sperm DNA, 250 ng of test DNA template (pA1i.o.CAT.CERE), 100 ng of internal control DNA, pb1(–41/+8).CAT+ and, 15 µM of Bio2.2 nuclear extracts. Reactions were performed as described previously (29), and the transcription products were detected by primer extension using a CAT gene-specific primer complementary to the 53 first nucleotides of the CAT gene.

RESULTS

cis-Acting Elements on the io Proximal Promoter—The organization of the 5’-flanking region of the vtg A1 gene is shown in Fig. 1A. This gene contains two transcription initiation start sites, determined by two different TATA box-containing promoters positioned 1.8 kbp apart, termed i1 and io for the coding region proximal and distal initiation sites, respectively. The three imperfect estrogen response elements (EREs) are located at positions –663, –375, and –333, i.e. between the two promoters, but physically closer to the i1 site. Of these three ERES, only the two downstream elements have been shown previously to be functional in in vitro transcription assays using either whole or nuclear HeLa cell extracts. In addition, both
sites are estrogen receptor- and estradiol-inducible as determined by S1 nuclease analysis using i- and io-specific primers on RNAs extracted from ER-transfected and estradiol-treated B3.2 cells or from liver of estradiol-stimulated Xenopus (20). However, no ERE has been detected up to 2 kbp upstream of the io promoter. Importantly, in the transfection experiments, estrogen inducibility of the io promoter was demonstrated from a template that contains approximately 2 kbp of the A1 promoter (construct pA1(2195/16).CAT), i.e. comprising only 200 bp of sequence upstream of the io transcription start site (20).

As no ERE is present upstream of the io promoter, we were interested in examining the transcription factors that are responsible for the significant in vitro transcription activity observed from the upstream io promoter and also to understand the mechanism by which the estrogen receptor confers inducibility to this promoter.

Computer-aided analysis of the 200 bp immediately upstream of the io transcription start site revealed the existence of several potential cis-acting elements. Of these, the most evident are three GC-rich motifs situated between positions -1862 and -1848 (numbering of the positions is with respect to the major transcription initiation site i of the vtg A1 gene promoter). These GC motifs have sequences corresponding to the consensus Sp1 binding site. In addition, the position of these GC boxes with respect to the initiation site conforms to the criteria previously proposed for Sp1 sites in promoters (30). Of interest also is the presence of a PuF binding site (31) at positions -1928 to -1917, a sequence with good homology to the Drosophila hunchback binding site (32) at positions -1896 to -1882 and a CCAAT factor (NF-Y) binding site (33) at positions -1939 to -1930 (see Fig. 1A).

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An Activity from Xenopus Nuclear Extracts Recognizes the GC Boxes of the io Promoter—First, we determined whether the sequence of the io promoter, containing the three GC-rich motifs, is recognized by Sp1 or other DNA-binding protein factors present in Xenopus-derived nuclear extracts. Thus, nuclear extracts either from B3.2 or from Xenopus female hepatocytes were tested by EMSA using as

![Image](https://example.com/image.png)
probe a 64-bp (Sp1-24) fragment containing the GC-rich sequences of the io promoter. As shown in Fig. 1, nuclear extracts either from B3.2 cells (Fig. 1B), or from hepatocytes (Fig. 1C), contain an activity that specifically binds to the 64-bp probe, forming the Ca complex in gel retardation assays. The binding to the probe was efficiently competed by an excess of either unlabeled Sp1-24 fragment or by an unlabeled fragment (Sp1-CAT) containing the five GC boxes from the SV40 early promoter, which are known to bind Sp1 (34). As a control, the ESV40 fragment that contains no GC boxes was not able to compete for the Ca complex, even at a 250-fold molar excess (Fig. 1, B and C). In contrast, the nonspecific complex, Cx, which appears only with the B3.2 extracts, was efficiently competed with the ESV40 fragment (Fig. 1B). Faster migrating minor complexes that appear to be specifically competed by GC box containing DNA sequences (Fig. 1, B and C) could represent either degradation products of the major binding activity (Ca) or distinct GC box-binding proteins. Specific binding of the nuclear extract activity to the GC sequences of the Sp1-24 fragment was demonstrated by methylation interference experiments. As shown in Fig. 1D, methylation of G residues in the proximal GC box of the io promoter prevents formation of the Ca complex. Consistently, methylation of the same residues did not influence formation of the nonspecific Cx complex observed with the B3.2 cell extracts (not shown). Thus, we conclude that Xenopus nuclear extracts possess an activity that can specifically recognize the proximal GC box of the io promoter. Based on the G residue methylation interference pattern, which is the same to that observed with purified Sp1 or with Sp1 from HeLa nuclear cell extracts (see below), we conclude that this activity is most likely Sp1. Additional evidence in favor of this conclusion is provided by the observation that the binding activity in Xenopus hepatocytes did interact with a Sp1 binding site even when the site is CpG-methylated (Fig. 2, A and B), a property characteristic of Sp1 (Fig. 2B and Ref. 35). In contrast, as was shown previously Sp1 binding is strongly reduced on binding sites in which some guanines have been replaced by O6-methylguanines (Fig. 2B and Ref. 36). More importantly, this is also confirmed for the Sp1-like binding activity from Xenopus hepatocyte nuclear extracts (Fig. 2C). We further characterized the biochemical properties of the binding activity in Xenopus hepatocytes by assessing its specific requirement for zinc to bind DNA, using a metal reconstitution assay. When Sp1 protein is treated in vitro with EDTA and then challenged with different metal sulfate salts, it recovers its DNA binding activity when zinc is added, but not when cobalt and nickel are added (Fig. 2D and Ref. 37). The Sp1-like activity in Xenopus hepatocyte nuclear extracts presents an identical behavior (Fig. 2D). Thus, in the several tests presented so far, the analyzed activity from Xenopus nuclei, which binds to Sp1 sites, has characteristics identical to those of purified Sp1. Therefore, we conclude that, most likely, this activity is Xenopus Sp1.

Purified Sp1 Binds to the GC Boxes of the io Promoter—Further confirmation of the function of the io promoter GC boxes as Sp1 binding sites was provided by the use of purified Sp1 protein in DNase I footprinting and methylation interference experiments (Fig. 3). In the DNase I footprint of labeled Sp1-24 probe, Sp1 protected two regions corresponding to positions −1856 to −1847 and −1884 to −1870 of the promoter (Fig. 3A). As shown in Fig. 1A, this region includes the three GC boxes of the io promoter. In gel retardation assays, binding of Sp1 to the Sp1-24 probe results in two specific complexes Cx and Ch, for the faster and slower migrating complexes, respectively (Fig. 3B). Probes partially methylated and labeled at either strand were used in EMSA with Sp1. The bound, modified probe from the above Ca and Cb complexes was isolated and subjected to piperidine cleavage. As shown in Fig. 3B, G residue methylation in the bound probe results in the proximal GC box in the case of the Ca complex. Similarly, with the probe isolated from complex Cb, which apparently results from multiple Sp1 molecules binding to all three GC boxes of the io promoter (Fig. 3B), methylation was observed only in the periphery of these sequences. As expected, activity present in HeLa cell nuclear extracts interacted with the Sp1-24 probe resulting in an EMSA complex that could be supershifted with an antibody directed against human Sp1 (Fig. 3C, left panel). Methylation interference experiments revealed that the HeLa Sp1 activity interacts with the proximal GC box of the io promoter (Fig. 3C). It is important to note that the same methylation interference pattern is observed on this sequence irrespective of the origin of the binding activity, i.e., nuclear extracts from Xenopus or HeLa cells, or purified Sp1 (compare Figs. 1D, 3B, and 3C). From the above it is concluded that Sp1, purified or from HeLa nuclear extracts, is able to recognize and specifically bind to the GC boxes of the io promoter. In addition, the activity present in the Ca EMSA complex from either hepatocyte or B3.2 nuclear cell extracts (Fig. 1, B and C) is most likely the Xenopus homolog of Sp1, as already demonstrated above. The human Sp1 monoclonal antibody could not cross-react with the Xenopus activity either in EMSA or Western blots (not shown), suggesting that the sequence corresponding to the antigen epitope is not well conserved between these two species. This is not surprising considering that the antibody is directed against an 18-residue-long peptide fragment of the human Sp1 protein.

Sp1 Can Activate Transcription from the io Promoter in Vitro—Binding of transcription factors to their cognate sites on a promoter is not sufficient to imply activation of the promoter by this factor. To ascertain that Sp1 is involved in the transcriptional activation of the io promoter, we employed two different experimental approaches. First, we performed transient transfection experiments in the Drosophila-derived cell line SL-2 with a reporter plasmid containing the io promoter cloned upstream of the CAT gene coding sequence (construct pio.CAT, see scheme in Fig. 4A) and a Sp1 expression vector. The choice of this particular cell line was justified, because, unlike the mammalian-derived cell lines where Sp1 is ubiquitous, it lacks endogenous Sp1 activity (8). As shown in Fig. 4A, the activation of the io promoter, as measured by the level of CAT activity, can be amplified by at least 20-fold when the reporter plasmid is cotransfected with the Sp1 expression vector. In contrast, the basal activity of the vtg B1 gene proximal promoter (promoter positions −41 to 8), that does not contain Sp1 sites (38), does not exhibit a similar Sp1-dependent activation (not shown, see below). The above strongly suggest that activation of the io promoter is Sp1-dependent.

The second approach consisted of an in vitro transcription assay in which the reporter plasmids that were used in the transfection assay, i.e., pio.CAT and the vtg B1 control plasmid (promoter positions −41 to 8), were used as templates of transcription. Transcription from the above templates was carried in B3.2 cell nuclear extracts in the presence or absence of purified Sp1 protein. As can be seen in Fig. 4B, increasing amounts of Sp1 resulted in strong induction of transcript synthesis from the io promoter but not from the control (ic), the truncated vtg B1 promoter. That the Sp1 effect was indeed mediated through the GC boxes of the io promoter was established by competition experiments using specific and nonspecific oligonucleotide competitors. Indeed, inhibition of transcription was observed when a 375 molar excess of the 60-bp fragment containing the 5 GC boxes of the SV40 promoter was used, while an equal concentration of a double-stranded ERE
Synergism between the Estrogen Receptor and Sp1

A
Wild-type probe (−1863/−1840):

CpG methylated probe (mC probe):

TACCCCTACCCCTACCC

GTTTTCCTACTGCTACCC

6-guanine methylated probe (mG probe):

CATACTGCTACCCCTACCC

GTTTTCCTACTGCTACCC

C
Purified Sp1

Probes: wt mC mG PP PP PP

Competitor: − − − wt mC mG wt − mG wt − mG wt − mG wt − mG wt − mG wt − mG wt

100X excess

D
Hepatocytes

Dialysis

Treatment

E
Purified Sp1

Dialysis

Treatment

1234567

FIG. 2
oligonucleotide, or a sequence containing the replication origin of adenovirus 2, did not lower the transcription levels of the \textit{i}o promoter. The modest increase in transcriptional activity observed in the presence of the non-specific competitors (compare lane 3 with lanes 6 and 7 in Fig. 4B) is likely to be the result of a titrating out of DNA-binding proteins that interact in a non-specific manner with the \textit{i}o promoter. Release of these non-specific interactions would then allow better binding of Sp1 to the promoter and, thus, increase transcription. This \textit{in vitro} assay was also used as an indirect demonstration of the involvement of the B3.2 endogenous Sp1 activity, which was detected with the EMSA (see Fig. 1B), in transcriptional activation. Transcription reactions were performed in the absence of purified Sp1 and with increasing amounts of the 60-bp SV40 promoter fragment able to titrate out the endogenous Sp1. As shown in Fig. 4C, \textit{i}o transcript inhibition was proportional to the amount of competitor present in the assay, which however, did not affect the control promoter. Furthermore, transcription from the \textit{i}o promoter in extracts depleted from the GC box binding activity could be restored by the addition of purified Sp1 protein (Fig. 4D). The above results demonstrate that both in transient transfection experiments as well as in \textit{in vitro} transcription assays, Sp1 is necessary and sufficient to stimulate transcription from the \textit{i}o promoter.

**ER and Sp1 Synergize for the Activation of the \textit{i}o Promoter**

S1 nuclease analysis has been previously used to demonstrate that the activity of the \textit{i}o promoter is ER-inducible when in the entire vtg A1 gene promoter context (20). This result suggests that ER bound to the ERU may be involved in the regulation of the upstream \textit{i}o promoter. Since the ERU is relatively far downstream of \textit{i}o, the involvement of ER in the activation of the \textit{i}o promoter could be explained if functional interactions of ER with transcription factors, i.e. Sp1, bound close to the \textit{i}o initiation site could occur. Thus, we explored this possibility. Using the Drosophila-derived SL-2 cell line, we have recently demonstrated that the nuclear hormone receptors PPAR and RXR can functionally interact with Sp1 for the transcriptional regulation of the ACO gene promoter (15). Therefore, we have used the same approach to investigate potential interactions of ER with Sp1 bound at \textit{i}o.

We utilized the pio.CAT.ERE reporter in which the \textit{i}o promoter is separated from the ER by the CAT gene coding sequences (1.6 kbp) and, thus, in that aspect, corresponds to the topological arrangement of the ER and Sp1 binding sites on the native A1 promoter (see scheme in Fig. 5A). The pio.CAT.ERE reporter plasmid has only a weak basal activity, when transfected in SL-2 cells in the absence of both ER and Sp1 expression vectors. Similarly, expression of ER alone has negligible effect on CAT transcription (Fig. 5A). In contrast, expression of Sp1 results in significant induction of CAT activity that is, nevertheless, hormone-independent. When both ER and Sp1 are expressed, a strong synergistic effect is observed between them, in the presence of 17\textbeta-estradiol, which results in CAT activity 4-fold higher than that of Sp1 transfected alone (Fig. 5A). Thus, the ER function as an activator depends on the presence of bound Sp1 to the \textit{i}o promoter, implying that functional interactions between the two proteins are essential for high transcriptional activity from the vtg A1 \textit{i}o promoter.

Functional interactions between ER and Sp1 were also demonstrated on another reporter construct (ERE.ACO.LUC, see scheme Fig. 5B) where an ERE is inserted upstream of the first four Sp1 sites in the proximal region (position −208) of the TATA-less ACO promoter (15, 21, see scheme in Fig. 5B). As in the above case, a similar estradiol-dependent, superactivation of transcription is observed from this promoter when both ER and Sp1 are present, reaching a level 6-fold higher than that obtained with Sp1 alone (Fig. 5B). However, in contrast to the pio.CAT.ERE reporter, a weaker ER and estradiol-dependent stimulation of the ERE.ACO promoter is obtained in the absence of Sp1 (Fig. 5B, inset). The obvious difference between these two reporter constructs is the position of the ERE with respect to the transcription initiation site. In the case of ERE.ACO.LUC the ERE is situated 208 bp upstream of the transcription initiation site, while it is situated 1.7 kbp downstream of \textit{i}o (4.7 kbp upstream) in the case of pio.CAT.ERE. Thus, it appears likely that, depending on the distance separating the ERE from the transcription initiation site, ER/Sp1 synergy confers estrogen inducibility (see pio.CAT.ERE) or strongly amplifies a weak ER response (see ERE.ACO.LUC).

\textit{Sp1}, Bound to the \textit{i}o Promoter, Is Not Involved in the Transcriptional Activity of the Downstream \textit{i} Promoter—Although the above results clearly demonstrate that ER and Sp1 synergize for the activation of the \textit{i}o promoter and of the hybrid ERE.ACO.LUC promoter, they do not address the question of whether this synergism affects the \textit{i} promoter activity as well (see Fig. 1A for location of the \textit{i} promoter).

To test this possibility, different A1 promoter-driven reporter plasmids were constructed in which the \textit{i}o promoter was either deleted or the intervening sequences between the ERU and the \textit{i}o promoter were eliminated (Fig. 6A). These constructs were used for transient transfections in HeLa cells in the presence or absence of estradiol and the ER expression vector. The results of these transfection experiments are summarized in Fig. 6B. The pA1 (−1995/+6).CAT construct (\textit{I} in Fig. 6A), that contains both the \textit{i} and \textit{i}o promoters as well as the intervening sequences between the two sites, is active and inducible by ER in the presence of estradiol. Similarly, the pio.TATA.i.CAT construct (\textit{II} in Fig. 6A), in which the intervening sequences between the \textit{i}o promoter and the ERU of the \textit{i} promoter have been deleted, is inducible by estradiol and ER and the induction factor (5-fold) as well as the absolute CAT activity, are equivalent to those observed with the pA1 (−1995/+6).CAT
reporter plasmid (Fig. 6B). Thus, positioning of the io promoter proximal to the ERU does not influence the transcriptional activity of the i promoter. Construct pio.GC.i.CAT (construct III in Fig. 6A), that lacks the sequences between position -580 and the Sp1 binding sites of the i promoter, was used to establish whether the presence of a TATA box between the ERU and the Sp1 sites is restrictive to interactions between the two activators. In addition, the Sp1 sites are closer to the ERU in this construct. Again, with this construct, ER/E2-dependent transcriptional activity and induction were similar to the pA1(-1995/+6).CAT and pio.TATA.i.CAT constructs. Finally, the ER/E2-dependent induction profile from construct pA1(-580/+6).CAT (construct IV in Fig. 6A), that lacks the sequences upstream of position -580, was similar to that observed with constructs pA1(-1995/+6).CAT, pio.TATA.i.CAT, and pio.GC.i.CAT (Fig. 6B). These similar activation profiles indicate the absence of synergistic interactions between ER and Sp1 for the activation of the i promoter, in contrast to what

**Fig. 3.** Purified Sp1 binds on the GC boxes of the io promoter. A, DNase I protection of a DNA fragment spanning the io promoter initiation site (positions -1995/-1763, see “Materials and Methods”), in the presence of purified Sp1. Lanes G, C/T, and F are the same as described in the legend to Fig. 1C. The positions of the protected sequences are indicated. B, the EMSA complexes, Ca and Cb, obtained with purified Sp1 protein and the Sp1-24 probe and methylation interference of the coding and noncoding strands of these two complexes. On the EMSA autoradiogram the supershift (SS) of the two complexes by the anti-Sp1 monoclonal antibody is indicated. On the methylation interference autoradiograms, lanes G, C/T, and F are the same as described in the legend Fig. 1C. The regions and G residues (black dots) that interact with Sp1 as well as the composition of the two complexes are indicated. Numbering of regions and residues is with respect to the vtgA1 promoter sequence. C, EMSA and methylation interference analysis of the endogenous activity from HeLa nuclear cell extracts (NCE) that interacts with the Sp1-24 probe. In the EMSA autoradiogram, the major complex, Ca, can be supershifted with the anti-Sp1 monoclonal antibody. The methylation interference pattern of the Ca complex is identical to that obtained with purified Sp1 protein (B) or to that of Xenopus hepatocyte nuclear extracts.
we have observed above for the io promoter. This does not seem to be a per se sequence context-mediated event but rather reflects the requirements for proper topological arrangement of trans factors on the promoter. Thus, it appears that binding of Sp1 upstream to the ER, that is at a distance from the io promoter, is not a favorable configuration for functional interactions between the two transcription activators for io promoter stimulation.

**DISCUSSION**

In this study we have used in vitro assays as well as transient transfection experiments to identify the cis- and trans-acting elements that control transcriptional activity from the io promoter, the upstream promoter of the Xenopus vtg A1 gene. The in vitro transcription assay, coupled to EMSA and methylation interference assays, demonstrated that the GC-rich sequences, in the proximity of the TATA box of this promoter, serve as binding sites for either purified Sp1 protein or for an endogenous Xenopus activity with similar properties. Presently, Sp1 or other members of the Sp family from Xenopus have not been characterized and therefore, we cannot speculate on the interspecies structural differences of these transcriptional activators. However, several lines of evidence suggest that the activity present in the Xenopus-derived nuclear extracts, able to recognize the GC boxes of the io promoter, is the homolog of Sp1. Previous studies (39) have shown that the G residues of the sequence 5'-GGGCG-3', i.e. four on the guanine-

**FIG. 4. Sp1 activates transcription from the io promoter in SL-2 cells and in vitro.** A. Drosophila SL-2 cells were transfected with the pio.CAT reporter plasmid, schematically represented on the top of the figure (squares represent the GC boxes, the oval represents the TATA box, and the arrow represents the transcription initiation site), in the absence (pPADH) or presence of the Sp1 expression plasmid (Sp1) as indicated. The average, with standard deviation, of three independent experiments is shown. B, in vitro transcription assay from the plasmid pio.CAT (io, 115 nucleotides) and the control vtg B1 promoter (ic, 89 nucleotide), in the presence of B3.2 nuclear extracts and increasing amounts of purified Sp1 (lanes 1–4). Transcription from io is inhibited in the presence of a 375 molar excess of the specific competitor Sp1-CAT double-stranded oligonucleotide (lane 5), but it is not affected by the presence of the nonspecific ERE and Ad1 DNA fragments (lanes 6 and 7, respectively). C, the endogenous Xenopus Sp1-like activity stimulates transcription from the io promoter in the absence of purified Sp1 (lane 1). B3.2 nuclear extract-dependent transcription from the io promoter can be inhibited with increasing amounts of the Sp1-CAT DNA fragment (0.6, 0.12, 0.24, 0.48, 0.96, 1.9, 3.8, and 7.6 pm, lanes 2–9, respectively). M is the molecular marker lane; io and ic are the same as in B. D, purified Sp1 protein (10 or 30 ng, lanes 3 and 4, respectively) restores transcription from the io promoter in B3.2 nuclear extracts that have been depleted of the GC box binding activity by the addition of 14 pm of the Sp1-CAT DNA fragment (lane 2). Lane 1 represents transcription from the io promoter due to the endogenous Sp1-like activity of the B3.2 nuclear extracts.
rich strand and one on the cytosine-rich strand, are the major sites of Sp1-GC box interactions. Our methylation interference experiments establish that the \textit{Xenopus} activity interacts precisely with these residues of the GC boxes of the \textit{io} promoter. Furthermore, like Sp1 this \textit{Xenopus} activity binds DNA even when the binding site is CpG methylated and, also like Sp1, it has a reduced binding when some guanines in the binding site are replaced by \textit{O}$_6$-methylguanines. Furthermore, it specifically requires zinc as a metal cofactor to efficiently bind its target DNA sequence, just like purified Sp1 (37). Consistent with these findings, induction of transcriptional activity from the \textit{io} promoter is dependent on the presence of purified Sp1 protein or on the endogenous \textit{Xenopus} activity in the \textit{in vitro} assay. Importantly, addition of purified Sp1 protein could restore transcriptional activity, specific to the \textit{io} promoter, in nuclear extracts depleted from all GC box-binding proteins. A further argument for the identity of the \textit{Xenopus} activity as Sp1 is that Sp3, the protein most closely related to Sp1 in terms of size, structure, expression pattern, and DNA sequence recognition (40), is a transcriptional repressor (41, 42). Our \textit{in vitro} transcription assay results clearly demonstrate that the \textit{Xenopus} activity has a stimulatory effect on transcription. The above conclusions are further supported by the results of transient transfections in a \textit{Drosophila} derived cell line devoid of endogenous Sp1-like activity. Indeed, we demonstrated that cotransfection of the Sp1 expression vector, along with a re-
porter plasmid under the control of the io promoter, was necessary and sufficient for basal transcriptional activity from this promoter.

Transient transfections in the SL-2 cell line were further used to explore the mechanism responsible for the previously observed ER-dependent transcriptional inducibility of the io promoter (20). Herein, we have demonstrated that this effect is mediated through functional synergism between ER and Sp1. Functional interactions between ER and Sp1 were also demonstrated on a TATA-less promoter consisting of the proximal ACO promoter, that contains four Sp1 binding sites, artificially fused to an ERE. Thus, such interactions are not restricted to promoters containing a TATA box. However, it is important to note that the topological arrangement of the cis elements appears to be an important parameter in determining the ability of a trans factor to act as an activator as well as in allowing functional interactions between different activators. Thus, on the promoter constructs studied here, the ER by itself cannot act as a transcriptional enhancer when the ERE is located at a distance from the transcription initiation site. However, binding of Sp1 to the proximity of the transcription initiation site converts the distally bound ER to a strong activator of transcription. In contrast, as the experiments with the ERE.ACO promoter demonstrate, Sp1 binding to the proximal promoter or the presence of a TATA box is not essential for ER-dependent activation when the ER is bound close to the transcription initiation site, but Sp1 dramatically amplifies the hormonal stimulation.

The function of Sp1 as an enhancer is still a matter of debate as conflicting results have been reported in the literature (43, 44). However, even in the case where the ability of Sp1 to act as a distal enhancer has been demonstrated, this effect is marginal and is dependent on high Sp1 concentrations. Our transient transfection experiments with reporter constructs in which the intervening sequence between the two promoters of the vtg A1 gene were deleted suggest that, at physiological
concentrations, Sp1 must bind proximal to the transcription initiation site to enhance transcription. In addition, binding of Sp1 close to the initiation site appears to be a requirement for positive interactions of this activator with the ER. This is also supported from our previous analysis of the ACO promoter where we had shown that the most distal (upstream) Sp1 binding site of this promoter does not contribute to the synergism between the PPAR/RXR heterodimer and Sp1 (15).

The nature of the interactions between ER and Sp1 that lead to functional synergism of these two transcription factors is not yet elucidated. It has been reported that Sp1 stabilizes weak interactions of ER with an ERRE half-site provided that this element is adjacent to a Sp1 site, as is the case in a number of naturally occurring promoters (45, 46), in contrast to the situation studied herein. However, direct contacts between the two proteins have not been observed even when they are bound to adjacent sites. Besides the reciprocal stabilization of the DNA binding abilities of the two proteins to their corresponding cis elements, at least three additional mechanisms could account for their synergistic effect on transcription. First, the documented interactions of ER and Sp1 with TAFII30 and TAFII110, respectively, may be responsible for the recruitment and stabilization of the RNA polymerase II holoenzyme on promoters that contain ER and Sp1 binding sites. Second, it is possible that ER and Sp1 interact with a common co-activator. Recruitment of this co-activator on the promoter could then result in increased affinity of the transcription initiation complex for this promoter. Finally, the established interaction of Sp1 with histone H3 (47) may contribute to chromatin reorganization in the vicinity of the io promoter, thus enhancing transcription from this site.

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