Sp1 Activates and Inhibits Transcription from Separate Elements in the Proximal Promoter of the Human Adenine Nucleotide Translocase 2 (ANT2) Gene*

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Expression of the adenine nucleotide translocator 2 (ANT2) gene is growth regulated. We report a feature of the ANT2 promoter that involves a novel regulatory function for the Sp1 transfactor. We show that expression from the ANT2 proximal promoter is modulated through three Sp1 elements, two of which activate and one of which partially inhibits transcription. The inhibitor site, box C, is juxtaposed to transcription start (nucleotides –7 to –2). Sp1 bound to box C decreases transcription initiation. This was demonstrated by introducing mutations in box C which (a) increased chloramphenicol acetyltransferase expression in the transient transfection assay and (b) inhibited binding of both purified Sp1 and Sp1 in crude nuclear extracts. The activating elements (A and B boxes) are located at adjacent sites in the distal region of the proximal promoter. Mutation of either box inhibits transcription by 90%, indicating that they act in a synergistic manner. Supershift experiments with crude nuclear extracts showed that only Sp1 was bound to the three GC boxes. The finding that Sp1 acts as an activator/inhibitor within the same promoter region was verified in NIH3T3, HeLa, JEG3, and COS-1, indicating that this dual effect of Sp1 is widely preserved. These data suggest a unique role for Sp1 and raise the possibility that growth activation of the ANT2 gene is regulated by the interaction of Sp1 on the A, B, and C boxes.

ANT2 is one of three genes that encodes mammalian adenine nucleotide translocator (ANT, ATP/ADP translocase) proteins (1–4). The three genes are differentially expressed in a tissue-specific manner (5–7), and during cellular differentiation (6–9). The ANT2 isoform is unique since it is expressed in a growth-specific manner (5–7), and during cellular differentiation (6–9). ANT2 cDNA clones were first isolated as one of which partially inhibits transcription. The inhibition from the proximal promoter is modulated through three Sp1 elements, two of which activate and one of which partially inhibits transcription. The inhibitor site, box C, is juxtaposed to transcription start (nucleotides –7 to –2). Sp1 bound to box C decreases transcription initiation. This was demonstrated by introducing mutations in box C which (a) increased chloramphenicol acetyltransferase expression in the transient transfection assay and (b) inhibited binding of both purified Sp1 and Sp1 in crude nuclear extracts. The activating elements (A and B boxes) are located at adjacent sites in the distal region of the proximal promoter. Mutation of either box inhibits transcription by 90%, indicating that they act in a synergistic manner. Supershift experiments with crude nuclear extracts showed that only Sp1 was bound to the three GC boxes. The finding that Sp1 acts as an activator/inhibitor within the same promoter region was verified in NIH3T3, HeLa, JEG3, and COS-1, indicating that this dual effect of Sp1 is widely preserved. These data suggest a unique role for Sp1 and raise the possibility that growth activation of the ANT2 gene is regulated by the interaction of Sp1 on the A, B, and C boxes.

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Experimental Procedures

Preparation of Clones and Oligonucleotides—The genomic clone of the human ANT2 gene (4) was a gift from Dr. J. Wurzel. A PstI/PstI fragment (nt –1237/+46, relative to transcription start (4)) from the 5’-flanking region of the gene was cloned ahead of the CAT reporter gene in pCATbasic (Promega). Additional clones (nt –674/+46, –253/+46, –87/+46) were prepared by removing specific restriction fragments from pCAT(–1237/+46) and religating the plasmid with DNA ligase. Fragments –64/+46, –87/+8, and –87/+8 with mutations in the A, B, and C GC boxes (see Fig. 2) were prepared by polymerase chain reaction amplification. All of the above fragments were cloned into pCATbasic. All clones were verified by DNA sequencing. Oligonucleotides used as electrophoretic mobility shift and supershift assay probes were synthesized (Fig. 2).

Cell Growth and Transfection—Human JEG3, HeLa, mouse NIH3T3 fibroblasts, and monkey COS-1 cell lines were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum (Flow Laboratories), 2 mM glutamine, 50 units of penicillin, and 50 μg of streptomycin/ml. The cells were plated in 175-cm² plastic flasks and grown to subconfluence.

The abbreviations used are: nt, nucleotide(s); CAT, chloramphenicol acetyltransferase.
Sp1 Inhibits Expression from the ANT2 Promoter

**RESULTS**

The ANT2 Promoter Is a Composite of Activation and Suppressor Regions—Transfection of deletion constructs of the human ANT2 promoter region (4) into human J E G3 choriocarcinoma cells, H ELa cells, and mouse NIH3 T3 fibroblasts (Fig. 1) reveals a complex promoter structure. Removal of nt –1237 to –674 (relative to transcription start (4)) decreases CAT expression by 30–50% in the three cell lines. More strikingly, removal of nt –674 to –235 results in reactivation of the CAT expression in the three cell lines. CAT expression supported by clone –235/+46 is 2–6-fold higher than that supported by the full-length promoter fragment (–1237/+46) in the different cell lines tested and 3–13-fold higher than that supported by clone –674/+46. These data suggest the presence of a negatively regulated element in the –674/+235 region which binds a factor(s) common to J E G3, H ELa, and NIH3 T3 cells. Ecniments in Fig. 1 indicate that the proximal promoter region, nt –235/+46, most probably contains the activating region of the promoter. This region, which provides maximum transcription activity, is characterized by the presence of 7 GC boxes (Fig. 2A). Removal of nt –235 to –87, containing 4 GC boxes separated by 20–40 nt, decreases CAT expression by approximately 50–60% in J E G3, H ELa, and NIH3 T3 cells (Fig. 1). The promoter activity of this shortened clone (clone –87/+46) was similar to or greater than that supported by clone –1237/+46. Further removal of nt –87 to –62, which includes two adjacent core Sp1 elements (Fig. 2, boxes A and B), resulted in an 80–95% decrease in promoter activity (relative to clone –235/+46) in J E G3, NIH3 T3, and H ELa cells. Thus, the activating region of the ANT2 promoter can be characterized as a GC rich region which binds a factor(s) common to these cell lines. The requirement exhibited by Sp1 for binding to its core element (19) fits with box C which is conserved in the proximal promoter region of the human ANT2 gene.

**Fig. 1. Analysis of the promoter region of the human ANT2 gene.** Fragments from the 5′-flanking region of the human ANT2 gene cloned ahead of the CAT gene in pCATbasic (Promega) were transfected for 24 h into human J E G3 and H ELa cells or mouse NIH3 T3 fibroblasts. Arrows, transcription start (+1); CAT activity was corrected for transfection efficiency and then normalized to the values obtained with fragment –1237/+46 in each cell line. All experimental points were run in triplicate. The mean ± S.E. (bars) is given for one to three separate experiments. Transfection was done by the calcium phosphate precipitate method. 2 × 10⁶ cells were grown for 24 h in 60-mm tissue culture dishes. Fresh medium was added 3 h before transfection. Cells were transfected for 24 h with 3 μg of reporter plasmid and 0.6 μg of pCH110 (Pharmacia Biotech Inc.) plasmid DNA. Medium was changed after the transfection period, and the cells were incubated for an additional 24 h. CAT and β-galactosidase activities (pCAT and pCH110, respectively) were measured according to the manufacturer’s instructions.

**Fig. 2. Summary of the proximal promoter region of the ANT2 gene.** The complete activating region (A) and the proximal region (B) of the human ANT2 promoter are shown. A, positions of the seven GC boxes relative to transcription start (+1) (arrow); B, Sp1-binding sites A, B, and C are boxed. Mutations in boxes A and B (see text and Fig. 3) are given under the wild-type sequence. Oligonucleotides (nt –13/8) carrying box C mutations 1, 2, or 3 (Mut-1, Mut-2, Mut-3) are shown. The same three mutations were also placed in box C within the context of the –87/+8 fragments (see Fig. 3).
on transfection of clones carrying C box mutations. In these
diplons, mutation of either the A or B box decreased CAT ex-
pression by greater than 95 percent (Fig. 3). Like the sup-
sershift experiments were conducted using an oligonucleo-
tide probe, but 25 ng was still not sufficient to shift probes
bearing mutated C boxes (not shown). (c) Nuclear extracts of
HeLa cells (Fig. 5) and NIH 3T3 cells (not shown) shift the
wild-type box C oligonucleotide, but not the mutated box C
oligonucleotides, indicating that protein is unable to bind to
the mutated oligonucleotide. (d) The low mobility band bound
to the wild-type box C oligonucleotide is supershifted by antibod-
ies against the Sp1 protein (Fig. 6). Antibodies against the Sp3
protein had no effect. The presence of Sp3 was tested since it
binds with the same apparent affinity to the Sp1 element (13,
14) but appears to suppress, rather than activate, transcrip-
tion (15, 21). This result was obtained in HeLa, JEG3, and
NIH3T3 nuclear extracts (Fig. 6).

The above experiments identify Sp1 as the major, and prob-
bly the sole, factor in several cell lines that binds to the C box.
To test if Sp1 also binds to the upstream A and B boxes,
supershift experiments were conducted using an oligonucleo-
tide probe, nt −87 to −58 (see Fig. 2), containing both boxes.
The major, low mobility band obtained with this probe was shifted
by antibodies against Sp1 but not Sp3 (Fig. 7). Antibodies
against the human AP2 protein were included since AP2 also
binds to a GC-rich element (22). AP2 antibodies had no effect.
The same results were obtained with nuclear extracts from
NIH3T3 and JEG3 cells (not shown). Thus, we conclude that all
three GC boxes bind primarily, if not exclusively, Sp1 in several
diverse cell lines.

Box C is Not Juxtaposed to a Putative Ets Site—The se-
quence TTCGC at positions −12 to −8 in Fig. 2 was origi-
nally reported as TTCGC (4). TTCGC introduces an opposite-strand
Ets binding site (GGAA) adjacent to box C. Ets-related proteins
regulate expression from the proximal promoters of other nu-
clear encoded OXPHOS genes (23–26). However, when we se-
quenced the ANT2 genomic clone received as a gift from J.
Wurzel (4), TTCGC was found as shown in Fig. 2. This se-
extence was subsequently confirmed using polymerase chain
reaction-amplified genomic DNA from human lymphocytes (not
shown). The lack of an Ets binding site is consistent with
studies reported above identifying Sp1 as the factor responsible
for modulating C box activity.

**DISCUSSION**

Sp1 is a ubiquitously expressed nuclear factor that is re-
quired for activation of a large number of regulated and con-

| C box sequence | JEG3 | NIH3T3 | HeLa | COS1 |
|---------------|------|--------|------|------|
| Wt (CCGCC)    | 100  | 100    | 100  | 100  |
| Mut-1 (CCCAC) | 576 ± 50 | 207 ± 12 | 268 ± 17 | 285 ± 46 |
| Mut-2 (CTGCC) | 1108 ± 108 | 664 ± 83 | 746 ± 73 | 519 ± 83 |
| Mut-3 (CACACC) | 630 ± 127 |        |      |      |
stitutively expressed genes. To our knowledge, Sp1 is reported to function only as an activator of transcription. Data reported in this paper on the proximal promoter of the human ANT2 gene raise the possibility that Sp1 may also interfere with transcription. Furthermore, Sp1 appears to have a dual function as an activator/suppressor in this promoter. This unique response to Sp1 is mediated through an octanucleotide (CCGC-CCCG) organized as a distal, direct inverted repeat that is required for transcription activation (Fig. 2, boxes A and B), and as a single proximal element that binds Sp1 and decreases transcription efficiency (Fig. 2, box C). These three elements appear to define the proximal promoter.

Transcription is strongly dependent on the inverted repeat A and B boxes. Replacing the CCGCCC core sequence with CATGACG, even in the box decreased CAT expression by more than 90%, indicating that the two boxes interact in a synergistic manner. This result is consistent with earlier reports that adjacent GC boxes are used to synergize Sp1-dependent activation (20, 21). DNA-bound Sp1 forms higher order homomeric complexes that provide interacting surfaces between complexes on adjacent elements (20). The Sp1 protein regions necessary for superactivation have been mapped, and a requirement for three domains, A, B, and the carboxyl-terminal D domain, has been demonstrated (20, 27–29). Although we have no direct proof that the same three domains of Sp1 participate in synergistic activation of the ANT2 promoter, we have shown by supershift experiments that Sp1 is the major, and most probably the only, protein bound to the A and B boxes. The presence of another candidate activating protein, Sp4 (13), was not tested. However, unlike Sp1, Sp4 does not synergize transcription from two adjacent Sp1-binding elements (21).

Several lines of evidence support the conclusion that box C is involved in the partial suppression of transcription from the ANT2 promoter. (a) Three mutations introduced into the core Sp1 element (CCGCC) of box C were all found to enhance transcription severalfold in transient transfection experiments. (b) The same mutations also prevented binding of nuclear extracts and purified Sp1 in mobility shift experiments. (c) No new protein-binding sites were created by the mutations, thus the elimination of binding sites is responsible for activation. Although the involvement of additional, low abundant factors cannot be entirely eliminated, supershift and competition gel shift experiments demonstrate that Sp1 is the major, if not only, nuclear factor that binds to box C. Similar results were
obtained with all of the above assays using human, monkey, and mouse cell lines, indicating that the mechanisms of activation and suppression of the ANT2 proximal promoter are widely preserved, an observation consistent with the ubiquitous nature of Sp1.

The above experiments raise the possibility that Sp1 can, in special cases, down-regulate transcription via a mechanism that is fundamentally different from Sp1-related suppression mechanisms described previously. Sp3, a member of the Sp1 family (13, 14), is a suppressor protein (15, 21, 30) that competes directly with Sp1 for binding to the Sp1 element (13). However, the A-, B-, and D-transactivating domains of Sp3 cannot replace the counterpart domains in Sp1 and appear to be nonfunctional (15). G10BP, a protein that inhibits Sp1 activation of the fibronectin gene, also acts via a similar mechanism, i.e., competition for Sp1 binding element (31). A second mechanism by which Sp1 function is suppressed has been described for the inhibitor proteins Sp1-I (16) and the ribonucleoprotein-like p107 (17), both of which form complexes with Sp1 and prevent Sp1 binding to DNA. In the above cases, transcription suppression is caused by interference with Sp1 activation. Our data point to a third, unique mechanism in which bound Sp1 itself decreases transcription initiation. The mechanism by which this is achieved is not clear. An attractive explanation is that Sp1 bound at the transcription initiation site decreases the efficiency with which the transcription machinery is recruited and assembled. The TFID complex protects an extended area on both sides of transcription start (32), and box C (nt −7 to −2) most probably lies within the initiator region.

Although our data demonstrate that Sp1 lowers transcription efficiency when bound to box C, it remains to be tested whether binding and release of Sp1 from the C box is a physiological mechanism for modulating ANT2 expression. ANT2 is actively expressed in growth-activated cell lines (6, 7, 8, 9, 11, 12, 33). Furthermore, activation of ANT2 expression is reportedly insensitive to cycloheximide (8), suggesting the involvement of a cell surface receptor-activated signal system. In line with this, phosphorylation of Sp1 has been reported in both human (34) and rat (35) nuclear extracts. Phosphorylation of HeLa cell Sp1 did not, however, alter Sp1-binding affinity or in
vitro transcription rates (34). By contrast, phosphorylation of rat Sp1 decreases binding (35). In the context of a Sp1 binding/release model suggested by our present findings, phosphorylation of Sp1 would be expected to decrease binding to the C box.

The ANT2 promoter is a complex structure with a suppressor region and multiple activating regions. The possibility that these regions can influence the interaction of Sp1 with the A, B, and C boxes remains to be studied. The GC rich –235/–88 activating region is particularly important in this respect since electron microscopic investigations have revealed interactions between distal and proximal Sp1 multimeric complexes through DNA looping (36). Furthermore, widely separated Sp1 binding elements can also participate in superactivation (20). However, removal of the distal GC elements (–235/–88) from the ANT2 promoter decreases CAT expression by only 50%. Thus, although the distal GC elements contribute to transcription efficiency of the ANT2 gene, the data do not support their involvement in superactivation. Even so, future experiments should test whether Sp1 bound to a distal GC element can cooperate with either the A or B elements to induce superactivation, or influence Sp1 binding to the C box.

In summary, we demonstrate that Sp1 binding to three GC elements in the proximal promoter of the human ANT2 gene, the data do not support their involvement in superactivation. Even so, future experiments should test whether Sp1 bound to a distal GC element can cooperate with either the A or B elements to induce superactivation, or influence Sp1 binding to the C box.

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