Characterization of a Silencer Element and Purification of a Silencer Protein That Negatively Regulates the Human Adenine Nucleotide Translocator 2 Promoter*

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Expression of adenine nucleotide translocator isoform 2 (ANT2) is growth regulated. In the present study, we report the presence of a silencer region in the human ANT2 promoter and the purification of a two-component factor that recognizes a specific hexanucleotide element, GTCCTG, of the silencer. Transfection of deletion constructs shows that ANT2 silencer activity extends over a region of at least 310 nts. However, mutating the GTCCTG element completely relieves silencing activity in the context of the human ANT2 promoter. The data suggest that the GTCCTG element might be required for maintaining silencer activity of the extended silencer region. The ANT2 silencer region cloned in front of the herpes simplex virus thymidine kinase promoter confers nearly complete inhibition to the heterologous promoter. However, unlike the ANT2 promoter, mutating the GTCCTG element restores only partial activity to the herpes simplex virus thymidine kinase promoter. A protein complex consisting of two major polypeptides of 37 and 49 kDa was isolated from HeLa nuclear extracts by affinity chromatography using the GTCCTG element as the affinity resin. Cross-linking studies and Western analysis indicate that p37 binds DNA. p49 appears to be loosely associated with the p37/DNA complex but is necessary for strong binding of p37. Our data implicating the GTCCTG element directly in silencing of the ANT2 promoter, together with data from the literature reporting the presence of this element within the silencer region of several additional promoters, suggest a general role of the GTCCTG element in transcriptional silencing.

The adenine nucleotide translocator (ANT)1 proteins exchange cytosolic ADP for mitochondrial ATP, thereby playing an essential role in maintaining cell metabolism and growth. Recent findings also implicate ANT in the initiation of events leading to apoptosis (1). Mammalian ANT is encoded in three genes, ANT1, ANT2, and ANT3 (2–5), that are expressed in a tissue-specific manner (6). ANT1 mRNA is expressed predominantly in heart and skeletal muscle (7, 8) whereas ANT2 mRNA is expressed in a broad range of tissues (6, 7, 9), but predominantly those tissues that undergo rapid proliferation (6, 10). However, the ANT2 isoform is unique in that it is also expressed in a growth-dependent manner (11) in a wide variety of cell lines. ANT2 expression is low in quiescent cells and is substantially increased by factors that induce entrance into G1 and subsequent cell growth (11). ANT2 expression in serumsupplemented NIH3T3 cells is inhibited by actinomycin D but not cycloheximide (12), indicating that expression is regulated at the level of transcription but does not require new protein synthesis.

The physiological significance of expression of just the ANT2 isoform in a growth-dependent manner has not been delineated, although it is possible that slight kinetic differences in ADP/ATP exchange catalyzed by the isoforms (13) might provide the growth-activated cell with an energetic advantage. To understand the complex regulation of ANT2 expression, we have analyzed its promoter (14). Our findings show that it is a complex array of positive and negative activating regions, with the major activating sites residing in a tandem Sp1 AB element (14) in the proximal promoter. We also identified a unique Sp1 site adjacent to transcription start that suppresses promoter activity when occupied by Sp1 in mammalian (14) and Drosofila cells. A second negative acting region was also identified in the distal region of the ANT2 promoter (14). In the present study, we have characterized this region. We show that the negative acting element is a unique silencer region that is distributed over a region of about 300 nts, but whose silencer function is directed from an element containing a GTCCTG core sequence. We also report the purification of a two-component factor that binds to this element and appears to be responsible for maintaining silencer function.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa cells were grown to subconfluence in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal calf serum supplemented with 2 mM l-glutamine, 50 units of penicillin, and 50 μg/ml streptomycin. The cells (4 × 10⁵) were plated on 60-mm Petri dishes and transfected by the calcium phosphate method (15) using 5–5 μg of reporter plasmid DNA containing the chloramphenicol acetyltransferase (CAT) gene and 2–5 μg of control plasmid DNA (pSV-LacZ or pLac-ANT2; 235/+46). Enzymatic activities (CAT, β-galactosidase, and luciferase) were measured according to the manufacturer’s instructions (16).

Plasmid Preparation—Preparation of pCAT-ANT2(−647/+46) and pCAT-ANT2(−235/+46) was described previously (14). To construct pCAT-ANT2(−546/+46) and pCAT-ANT2(−413/+46), corresponding

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1 The abbreviations used are: ANT, adenine nucleotide translocator; HSVtk, herpes simplex virus thymidine kinase; CAT, chloramphenicol acetyltransferase; wt, wild type; mut, mutant; nt, nucleotide; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; GABP, GA-binding protein; NF1, nuclear factor-1.

2 Unpublished observations.

3 A. Zaid, R. Li, K. Luciakova, P. Barath, S. Nery, and B. D. Nelson, unpublished observations.
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ANT2 promoter fragments prepared by restriction enzyme digestion (BamHI/PstI and DraI/PstI, respectively) were cloned into pCATBasic (Promega). pCAT-ANT2(-647/+46)mut was constructed by the overlap-extension polymerase chain reaction (17). A mutated (CC to AA) oligonucleotide covering nts 338 to 318 (5'CCAAGATATGTAATGCCTCC-3') of the ANT2 promoter was synthesized and used as a primer for introducing the mutation into the −647/+46 fragment. The M13 (nts 2230–2208) and CAT (nts 2307–2255) primers of pCATBasic were also used. All other deletion clones containing mutated oligonucleotide −338/−318 were constructed by restriction deletion of pCAT-ANT2(-647/+46)mut. pCAT-ANT2(-546/−235)wt and pCAT-ANT2(-546/−235)mut, which were used as templates for the polymerase chain reaction to synthesize DNase I footprint probes, were created by removing the Smal/PstI (−235/+46 fragment from pCAT-ANT2(−546/+46)wt or pCAT-ANT2(-546/+46)mut, respectively, followed by T4 DNA polymerase blunting and plasmid re-ligation. All clones were checked by restriction enzyme digestion and sequencing. Heterologous promoter constructs of the ANT2 silencer region and the HSVtk promoter were prepared by cloning ANT2 promoter restriction fragments into the pBLCAT2 (18) plasmid. The clones were checked for correct orientation by restriction enzyme digestion, and the presence of mutation within the −338/−318 element was confirmed by sequencing.

Preparation of Nuclear Extracts—HeLa and NIH3T3 cells were grown to confluence in 175-cm² flasks. Cells were harvested, and nuclear extracts were prepared as described previously (19). Rat liver nuclei purified as described previously (20) were generously provided by Madeleine Kihlmark (Stockholm University, Stockholm, Sweden), and nuclear proteins were extracted in high salt C buffer as described previously (19). The protein was measured using the Bio-Rad Protein Assay (Bio-Rad). Nuclear extracts were stored in 20-μl aliquots at −70 °C.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA reaction mixture (20 μl) containing 20 mm K+ -Hepes (pH 7.9), 70 mm KCl, 10% glycerol, 5 mm MgCl₂, 2 mm dithiothreitol, 8 μM ZnCl₂, 1.5 μg of nuclear extract, 250 ng of poly(dI:dC)poly(dI:dC) (Pharmacia), and competitor DNA (where indicated) was incubated on ice for 10 min. Radioactively labeled oligonucleotide probe (10,000 cpm) was added, and the reaction was incubated for 20 min at room temperature. Bound complexes were separated on 5% native acrylamide gels in 0.5x TBE (1× TBE = 90 mm Tris, 90 mm boric acid, and 2 mm EDTA, pH 8.0). The gels were dried and autoradiographed.

DNase I Protection Assay—DNase I protection was performed as described previously (16). Radioactive probes were prepared by polymerase chain reaction using 5'32P-labeled CAT primer, M13 primer, and pCAT-ANT2(-546/−235)wt or pCAT-ANT2(-546/−235)mut as the templates. The probe (20,000 cpm) was partially digested by RNase-free DNase I (Boehringer Mannheim) in the presence or absence of HeLa nuclear extract (20 μg) and separated on a 6% acrylamide sequencing gel.

UV Cross-Linking and Southern Western Analysis—Radioactive oligonucleotide probes (ANT2−338/−318wt or ANT2−338/−318mut) were incubated with HeLa nuclear extract under the exactly described for the EMSA. Samples were placed 5 cm from a UV Transiluminator (Ultra-Violet Products, Inc.) and exposed to UV light for 10, 20, or 30 min. Subsequent DNase I/micrococcal nuclease treatment was omitted due to the 5' end-labeling of the probes. Crosslinked complexes were separated by 10% SDS-PAGE, dried, and autoradiographed. For Southernwestern analysis, increasing amounts of HeLa nuclear extract (10, 25, and 50 μg) were separated by 10% SDS-PAGE and electrobotted onto a nitrocellulose membrane. Membranes were treated with guanidine hydrochloride and incubated with radioactively labeled oligonucleotides as described previously (21).

Protein Purification—Nuclear extracts from 1.5 × 10⁹ HeLa cells were purified (22) and separated on Sephade G-100 SF column (Pharmacia) in a buffer containing 20 mM Hepes (pH 7.9) and 400 mM KCl. Eluted fractions were tested for protein binding to the ANT2−338/−318 oligonucleotide by EMSA. Active fractions were pooled and adjusted to 20 mM Hepes (pH 7.9), 100 mm KCl, 5 mM MgCl₂, and 10% glycerol. To remove nonspecific DNA binding proteins, the protein sample was electrophoretically incubated (1 h, 4 °C) with mutated ANT2−338/−318 oligonucleotide coupled to CNBr Sepharose 4B (Pharmacia) (23). The flow-through was collected and incubated (1 h, 4 °C) with 100 μl of immobilized streptavidin (Boehringer Mannheim) to remove streptavidin-binding proteins. Biotinylated ANT2−338/−318 oligonucleotide probe (850 pmol) was added, and the samples were incubated for 1 h at 4 °C. Fresh streptavidin resin (100 μl) was added, and the incubation was repeated. The resins with bound DNA and proteins were collected, washed in buffer containing 20 mM Hepes (pH 7.9), 5 mM MgCl₂, 10% glycerol, 0.1% Triton X-100, and 0.1 M NaCl, and eluted stepwise with the same buffer containing increasing concentrations (0.1–1 M) of NaCl. Finally, streptavidin resins were washed with 100 μl of 0.1 M glycine (pH 2.5).

RESULTS

Characterization of a Negative Regulatory Region in the ANT2 Promoter—We previously identified a region of the human ANT2 promoter between nts −647 and −235 (14) that exhibited negative regulation in three different transfected cell lines (JEG3, NIH3T3, and HeLa), suggesting the involvement of a factor(s) common to all of the cell lines tested. To further define this region of the ANT2 promoter, additional 5' deletion clones were constructed and tested in transient transfection experiments using HeLa cells (Fig. 1). Deletion of nts −647 to −546 had no significant influence on the promoter activity. By contrast, the stepwise deletion of nts −546 to −413 and nts −413 to −235 each resulted in an approximately 2-fold increase in ANT2 promoter activity. Thus, negative regulation is spread over an extended area of about 310 nts (nts −546 to −235). To emphasize the bipartite distribution of the negative acting region (Fig. 1), we shall refer to fragment −546/−413 and fragment −412/−235 as silencer regions A and B, respectively. Together, the A and B regions decrease ANT2 promoter activity by 70–75%.

Identification of DNA Binding Sites within the −546/−235 Region of the ANT2 Promoter—Protein binding sites on the silencer region were determined by DNase I protection using ANT2−546/−235 as the probe and HeLa nuclear extracts (Fig. 2A). This probe includes both the A and B silencer regions. However, DNase I-protected sites were found only in the B region (nts −413/−235). The most strongly protected area covers nucleotides −339 to −310 (region 2, Fig. 2A). The core sequence of this site consists of a hexanucleotide direct repeat (GTCCTG) with no spacing (Fig. 2B). Two additional weakly protected regions are located upstream and downstream from region 2. Region 1 is weakly protected over a 14-nt sequence containing a nuclear receptor half-site (AGGTTCA) on the noncoding strand. Region 3, which is located upstream from region 2, covers a 27-nt-long sequence and includes strong DNase I-hypersensitive sites. With the exception of a very weak correlation to the NF1 element, no conserved transfactor binding elements could be identified in region 3 by computer analysis (24).
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**Identification of a Specific GTCCTG Element Binding Activity**—To further study protein binding to region 2, a wild type oligonucleotide covering the central part of region 2 (nts –338 to –318) and a mutated oligonucleotide (CC to AA; Fig. 2B) of the same region were used in EMSA. Competition experiments showed that binding to the wild type probe was nearly eliminated by a 5-fold molar excess of the unlabeled probe but was unaffected by a 50-fold excess of the mutated probe (Fig. 3A). In addition, no binding was observed when the mutated oligonucleotide served as the EMSA probe (data not shown).

The region 2-binding protein is also present in mouse NIH3T3 cells and rat liver. Nuclear extracts from both sources exhibit specific binding to the wild type probe (Fig. 3B). Thus, the silencer protein appears to be widely expressed in mammalian cells.

As shown in Fig. 2A, three sites on the B silencer region are contacted by nuclear extract components. To determine whether region 2 influences binding to the weaker sites, the mutated region 2 sequence was placed in the context of the long promoter fragment (nts –546/–235). This construct was subjected to DNase I protection analysis with HeLa nuclear extract (Fig. 3C). As expected, protection of the mutated region 2 site is abolished, and, in addition, the hypersensitive bands that surround it are lost. However, protection of regions 1 and 3 is not significantly affected by a mutation in region 2, suggesting that protein binds to these sites independent of region 2.

The GTCCTG Element Is Required for Silencing ANT2 Promoter Activity—To determine whether the strongly protected region 2 could influence silencer activity, deletion clones bearing the mutated GTCCTG element were constructed and fused with the CAT gene. Promoter activities of these clones and the corresponding wild type clones were measured in transient transfection experiments (Fig. 4). Normalized CAT activity was increased up to 3.7-fold in the mutated clones, and, in the case of mutated fragments –546/+46 and –413/+46, promoter activity was restored to levels comparable to those obtained with clones in which the A and B silencer regions were deleted (clone –235/+46, Fig. 1). This observation indicates that the mutation in the GTCCTG element region 2 is able to eliminate silencing from the distal A suppressor region (nts –546/–413). Thus, the silencer protein appears to play a central role in organizing or maintaining the entire silencer region.

Silencing Activity Can Be Conferred to a Heterologous Promoter—Fig. 5A shows that silencing could be conferred to the heterologous HSVtk promoter in transfected HeLa cells. Silencing function was retained in the –413/–235 ANT2 promoter fragment that includes the GTCCTG element. Interestingly, silencing of the thymidine kinase promoter reached 87–95%, which was higher than that observed in 5′ deletion experiments with the native ANT2 promoter. As a control, the region immediately upstream of the ANT2 silencer (nts –647/–546) that lacks the GTCCTG element was also cloned in front of the thymidine kinase promoter and transfected into HeLa cells. This fragment exhibited no silencer properties (Fig. 5A). Mutated GTCCTG elements partially relieved the silencing of the heterologous ANT2-thymidine kinase promoter (Fig. 5B), but unlike the native ANT2 promoter, full restoration was not achieved.**

Characterization of a Specific Protein that Binds Region 2 of the Silencer—A computer search of a vertebrate transfactor database (24) failed to reveal a transfactor that specifically recognizes the region 2 sequence. To estimate the size of the protein binding to this DNA sequence, both cross-linking and Southwestern blot analysis were performed. Using HeLa nuclear extracts, a single polypeptide with an apparent mass of 33 kDa is cross-linked to the wild type ANT2 –338/–318 probe, but not to the mutated probe (Fig. 6A). No other specific signals were detected, even under prolonged UV light exposure (data not shown). Southwestern blot analysis with wild type ANT2 –338/–318 oligonucleotide as the probe also identified a single band that did not hybridize with the mutated probe (Fig. 6B). The apparent mass of this polypeptide was 37.9 kDa. Because the mobility of cross-linked polypeptides can be affected by a covalently attached DNA probe, we conclude that the molecular mass of the DNA-binding protein is most likely around 38 kDa. This is confirmed by an analysis of the purified factor (see below).

The presence of a single cross-linked band on SDS gels (Fig. 6A) suggests that the DNA-binding protein binds as a monomer, although the symmetric organization of the GTCCTG element is suggestive of a dimer binding site. However, we could not eliminate the possibility that the DNA-binding protein interacts with additional factors, because protein cross-
The DNA sequence of region 2 (noncoding strand, 5' -> CAG- GACCAGGAC-3') resembles a general binding site for the family of Ets proteins (5' -> C/AGGAA/T-3'); Ref. 25). However, antibodies against the conserved region of human Ets-1 protein (amino acids 362–374) failed to recognize the DNA-binding protein in an EMSA supershift assay (data not shown). This antibody (catalogue number sc-112X; Santa Cruz Biotechnology, Inc.) cross-reacts with several members of the Ets family.

DNA Affinity Purification of the Region 2-binding Protein from HeLa Nuclear Extracts—A protein that binds specifically to the GTCCTG element was purified from HeLa nuclear extracts by DNA affinity chromatography using a biotin-labeled region 2 oligonucleotide (ANT2 338/318) as the affinity ligand (Fig. 7A). The fraction from which protein was purified by the affinity chromatography step (Fig. 7A) had previously been treated with a DNA resin containing the region 2 oligonucleotide bearing a mutated GTCCTG element. Thus, the protein purified in Fig. 7A binds specifically to the wild type GTCCTG element. Fig. 7A also shows that the polypeptides were not retained on immobilized streptavidin in the absence of the ANT2 338/318 oligonucleotide ligand. Together, the above experiments show that the purified polypeptides associate specifically with the region 2 GTCCTG element.

The affinity-purified fraction contained two major polypeptides with molecular masses of 49 and 37 kDa that co-eluted from the affinity resin between 0.2 and 0.4M NaCl (Fig. 7A). Only residual binding of these polypeptides was observed to columns containing the mutated oligonucleotide ligand (data not shown). The apparent mass of the smaller polypeptide is identical to that of the DNA-binding protein identified with Southwestern blot analysis and agrees well with that observed by cross-linking. Based on these criteria, we conclude that p37 most probably binds DNA.

Although p49 appears to co-elute with p37 in Fig. 7A, in other experiments, proportionately more p49 is eluted at lower salt concentrations (0.2 M), suggesting that the p49 association or mut probes were digested by DNase I in the presence or absence of HeLa nuclear extract. Only the sequence of the B silencer region is shown, because no protection of region A was observed. The changes in the DNase I digestion pattern within region 2 are due to the introduction of the mutation (Fig. 2). The numbering of the protected regions corresponds to that in Fig. 2.

![Diagram](https://via.placeholder.com/150)
with the p37/DNA complex is weaker than the p37/DNA contact itself.

The functional integrity of the DNA affinity-purified protein was tested by EMSA using the ANT2 -338/-318 wt oligonucleotide (see above) as the probe (Fig. 7B). Fractions eluted from the affinity resin with 0.2 or 0.4 M salt containing both p37 and p49 gave a single, strongly shifted band identical to that obtained with crude nuclear extracts. CAT activity was normalized on transfection efficiency control. The values of the activities of mutated clones were set relative to the corresponding wild type clones. All data represent the mean value ± S.E. of three independent experiments. Each experimental point was done in triplicate.

**DISCUSSION**

In the present study, we describe a silencer region within the human ANT2 promoter and report the purification of factors that are essential to silencing. The silencer region extends over 310 nts (nts -546 to -235) and was divided by the transfection of deletion constructs into two regions, which were designated A and B. DNase I-protected sites are restricted to the B region, but both regions are required for complete silencing. Silencing by the A and B regions appears to be additive rather than synergistic. The combined A and B regions completely suppress the activity of the heterologous HSVtk promoter, showing that it functions as a general, position-independent silencer.

Although both the A and B regions are required for maximal silencing of transcription, the B region, and more specifically, one element within the B region, is required for the expression of the A region's silencing capacity. This element, encompassing 29 nts with a central, directly repeated hexanucleotide, 5'-GTCCTG-3', is the most strongly protected DNase I site within the B region. The central role of the hexanucleotide repeat is clearly shown by the finding that the mutated form (GTCCTG to GTAATG) abolishes silencer function. The GTCCTG element thus appears to organize the entire silencer region in a manner that is not yet understood. Indeed, the role of the A region remains an enigma. It is possible that specific suppressor proteins that are not detected by our methods bind to region A. If present, however, their association with DNA would probably be controlled by protein binding to the GTCCTG element. A second explanation might be that the silencer region includes a nucleosome assembly site. The A region contains putative AT tracts that are important in nucleosome assembly (27, 28). Nucleosome assembly is strongly influenced by specific DNA-binding proteins, including hormone receptors. Thyroid hormone and 9-retinoic acid receptor were reported to disrupt chromatin structure and facilitate tran-
scriptional repression in the absence of hormone by a mechanism that does not involve deacetylases (29). Region B of the ANT2 silencer is footprinted over a conserved hormone response element half site (AGGTCA), but this footprint is not altered by mutating the GTCCTG element that prevents silencing. Whatever the mechanisms of suppression through the A+B silencer region, it is clear that the factor binding to the GTCCTG element is of central importance.

A search of the eucaryotic promoter data base (GenBank) shows that hexanucleotide repeat (GTCCTGGTCTCG) is rarely found in mammalian promoters. By contrast, the hexanucleotide (GTCCTG) is present in a large number of promoters. In most cases, it appears to be part of an enhancing or activating element (30–33). However, in glutathione transferase P (34), apolipoprotein A1 (35), and the major histocompatibility complex class II HLA-DRA (36) promoters, this element is present within a silencer region. It is not clear, however, if the same proteins are responsible for silencing these and the ANT2 promoters. Thus, whether the GTCCTG element recognizes a specific silencing factor remains an open question.

The ANT2 GTCCTG element-binding protein isolated by us is composed of two major polypeptides of 37 and 49 kDa. Cross-linking studies show that only p37 binds DNA. p49 appears to be loosely associated with p37, and the two can be partially separated during chromatographic separation. However, p49 appears to strengthen the binding of p37, because chromatographic fractions containing predominantly p37 bind the DNA probe weakly. Thus, p49 appears to function as an ancillary protein to p37. This behavior is reminiscent of that displayed by members of the Ets family. For example, GABPs binding to DNA is greatly enhanced by GABPBs (37, 38), and Elk-1 (39) and Sap-1 (40) require contact with the serum response factor before contacting the serum response element. Nuclear respiratory factor-2 (41), a GABP family factor that functions as a transcriptional activator in the promoters of several OXPHOS genes (see Ref. 42 for a review), was recently described. Although the ANT2 silencer element contains repeated CCT motifs, which could form the core binding element of the GABP proteins, GABPα has a larger mass (58 kDa) (41) than the ANT2-binding protein (37 kDa). Furthermore, to our knowledge, there is no report that the GABP proteins function in any capacity than activating. Because of this, and because of our inability to induce supershifts with antibodies against general Ets proteins, we conclude that the ANT2 binding factors most probably do not belong to the Ets family.

Multiple forms of the NF1 family were recently reported to bind to the glutathione transferase P promoter silencer (43). Four major polypeptides were isolated in a complex that bound to the dominant GPS4 silencer element containing the GTCCTG element (43). These polypeptides ranged from 33 to 41 kDa, close to the mass of the ANT2 p37-binding protein. However, a conserved NF1 binding element could not be detected by a computer search of the ANT2 silencer. Thus, it seems unlikely that we have isolated members of the NF1 family, although this remains to be rigidly excluded. There are, however, several interesting similarities between the glutathione transferase P and ANT2 silencer regions: (a) both contain multiple protein binding sites, (b) mutation in single sites results in a significant increase in promoter activity, and (c) despite the fact that expression of the glutathione transferase P gene is restricted to tumor cells derived from the liver, silencer activity was observed with no tissue restriction (34). This correlates well with previous results from ANT2 transfection experiments (14) and with the observation that ANT2 GTCCTG binding activity is widely distributed in mammalian tissues (Fig. 3B).

A 36-kDa DNA-binding protein was isolated (44) that most probably binds to the human HLA-DRA Vbox silencer (36) that contains a GTCCTG element. This polypeptide has not been identified, and its relationship to the ANT2 p37 DNA-binding proteins is unknown. As discussed above, there are reasonably strong arguments that p37 might belong to a class of silencer proteins that have not previously been identified. In view of the apparent broad distribution of GTCCTG binding activity, it is important to identify p37 and establish its role in promoter silencing.

The role of the silencer region in expression of the ANT2 gene also remains to be delineated. The ANT2 gene was isolated as one of the early immediate genes during the growth stimula-
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tion of quiescent cells (11). Several laboratories demonstrated regulation of the ANT2 gene under different physiological conditions. ANT2 transcripts are up-regulated in activated T cells and various growth-activated cells (45–47) and are down-regulated in differentiating HL-60 cells (48), myoblasts (7, 8), quiescent cells (11), and cells approaching confluence (48). We recently identified a far upstream promoter region (~1237–546) that appears to be responsible for the down-regulation of ANT2 transcript during the growth of NIH3T3 cells to confluencia. However, the nature of this suppression and its mechanism is not known; therefore, we cannot exclude the interaction of this region with other elements within the ANT2 promoter including the silencer described in this work.

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