This work characterizes a 250-base pair (bp) fragment of the mouse carbonic anhydrase II (CAII) gene as an efficient but not cell-specific promoter. This fragment contains multiple consensus cis regulatory regions that interact in a complex fashion to regulate transcriptional activity from the promoter. Truncated fragments of the 250-bp promoter retain transcriptional activity. The 90-bp 5' “GC”-rich portion of the promoter can direct transcription independently from the consensus TATA box and also contains a silencer that diminishes transcription from the 3' 160-bp portion of the CAII promoter as well as from the SV40 promoter. There are two nonconsensus dual functioning AP2-like elements on the promoter that are essential for core promoter activity and cAMP-mediated increases in transcription of the gene. A nuclear protein of ~65 kDa binds to these elements and is present in nuclear extracts of nonstimulated and forskolin-stimulated NIH-3T3 cells. I conclude that these nonconsensus AP2-like elements and their cognitive binding protein play a major role in the expression of the CAII gene.

Carbonic anhydrase II (CAII) is an ubiquitous enzyme that catalyzes the series of reactions shown below. By virtue of its function CAII plays a role in the maintenance of intracellular pH and as such is fundamentally important for cell function (1).

\[
\text{H}^+ + \text{HCO}_3^- \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}_2\text{O} + \text{CO}_2 \quad (\text{Eq. 1})
\]

Although the genes for the mouse and human CAII have been cloned little is known about their transcriptional regulation (2–4). Venta et al. (2) have previously compared the 300-bp promoter of the human gene with a 250-bp portion of the mouse gene flanked by PstI-PstI restriction sites. These 5'-regions of the genes contain the polymerase cap sites and the TATAAA sequence but they are also extremely “GC”-rich giving them the appearance of “housekeeping” promoters. It has been speculated that methylation of the GC-rich islands may play a role in transcriptionally regulating the CAII genes. Specifically, the 250-bp PstI-PstI fragment of the mouse gene contains a consensus Sp1-binding site that is 150 bp upstream from the TATA box. An Sp1 site and TATA box are similarly arranged on the human gene but the distance between them is 115 bp. It has not been determined if the distance between the sites is critical for optimum function of either promoter. Additionally each gene contains a nonconsensus Sp1 site, GGGCCGCC, that is within 30 bp of the TATA box. An Sp1 site located in proximity to a TATA box usually enhances TATA-driven transcription but in other cases remotely located Sp1 sites, in multiple copies, or surrounded by GC-rich islands can activate transcription independently from the TATA box (5–7).

Such may be the case with the CAII promoter, adjacent to the consensus Sp1 site on the mouse gene is a second GC-rich zinc finger-binding site, GGGGGGGCCC, that is similar to the Krox-20 site, GCGGGGGGCG, which transcriptionally activates genes in response to serum (8, 9). With a similar arrangement that includes a GC-rich segment and a TATA box, the human triose-phosphate isomerase gene promoter can function in either a “TATA-independent” or “TATA-dependent” manner (6). In this light, the interactions between the GC-rich and TATAAA sequences on the mouse CAII gene may be dependent on cell-specific cis/trans interactions.

In addition to the sites described above, the mouse and human CAII genes contain two pyrimidine rich sequences that are similar to those on the rat prolactin gene (10) and the rat tyrosine aminotransferase gene (11). These sequences are thought to possess AP2-like (AP2-LE) function that includes basal, cAMP, and protein kinase C-mediated transcriptional activity, even though they do not share the AP2 binding sequence (CCCCAGGC) (12). Structurally these 2AP2-LE’s share more homology with the pyrimidine-rich, ACCCTTCCTAGTATCACCC, basal promoter elements of the adenovirus type 2 1 Va2 gene (13). The 10 bp encoding the two pyrimidine-rich AP2-LE segments on the mouse CAII gene differ only in the addition of a “T” to the 5’ site. These two sites lie within 14 bp of each other and the 3’-AP2-LE is 34 bp from the TATA box.

\[
5'-\text{AP2-LE 5'}-\text{CGGTCTCCTCCCCT}-3' \quad (\text{Eq. 2})
\]

\[
3'-\text{AP2-LE 5'}-\text{CGGCC-CCTCCCCT}-3' \quad (\text{Eq. 2})
\]

Computer search of >500 bp of the mouse CAII gene has not identified any consensus cAMP (CRE) or phorbol ester-responsive sites. Yet, Campbell et al. (14, 15) have shown that transcription of the CAII gene, in the gastric parietal cell, is regulated by cAMP and protein kinase C-mediated events. Therefore, the 2 AP2-LE elements may mediate these responses.

Thus, this 250-bp portion of the mouse CAII gene is rich in potential cis regulatory elements that may prove to interact in a complex fashion to confer constitutive or facultative transcriptional activity as well as cAMP and phorbol ester...
FIG. 1. CAII-pCAT constructs.
The SV40 promoter-enhancer plasmid was used to establish the optimum transfection conditions for each cell type. The other plasmids were used to test CAII promoter, enhancer, and CRE function. The plasmids were purchased from Promega.

<table>
<thead>
<tr>
<th>Site directed mutations</th>
<th>Mutant sequence</th>
<th>Mutant sequence</th>
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<tbody>
<tr>
<td>TATA</td>
<td>TATA</td>
<td>TTTT</td>
</tr>
<tr>
<td>5'-'Ap2-LE*</td>
<td>TCTCTCTCCCT</td>
<td>TCTCTCTCTCT</td>
</tr>
<tr>
<td>3'-Ap2-LE*</td>
<td>TCTCTCTCCCT</td>
<td>TCTCTCTCTCT</td>
</tr>
<tr>
<td>Zn finger</td>
<td>GCGGGGCCC</td>
<td>GCGGGGCCC</td>
</tr>
<tr>
<td>Sp1</td>
<td>CCCCGGCC</td>
<td>CCCCGGCC</td>
</tr>
<tr>
<td>Nonconsensus Sp1</td>
<td>GGCGGCCC</td>
<td>GAGCAGGAG</td>
</tr>
</tbody>
</table>

* This mutant called the 2-Ap2-LE mutant contained mutations at both sites. The oligo used contained the sequences shown above as well as the intervening bases on the fragment. We made constructs with the following multiple site-directed mutations: 2-Ap2-LE and TATA box (2-Ap2-LE/TATA), Sp1 and Zn finger (Sp1/Zn), and Sp1/Zn and 2-Ap2-LE (Sp1/Zn/2-Ap2-LE). We also mutated the TATA box on the 160-bp TATA domain (TATA/TATA D).

responsiveness. I chose to explore the interaction of these sites on the PstI-PstI fragment of the mouse gene and to characterize the function of this promoter.

MATERIALS AND METHODS

Sequencing

The 250-bp portion of the mouse CAII gene was sequenced in both orientations using the method of Sanger et al. (16). The fragment was sequenced in the pGRM-4 blue plasmid using the Sp6 and T7 promoter primers and subcloned into the M13 mp19 plasmid and sequenced using universal primers. Likewise, the sequence of all the site-directed mutations and truncated fragments made from the 250-bp fragment was confirmed.

Cell Lines and Transfection

I chose to use NIH-3T3, mouse embryonic fibroblasts, for our studies because they are easily transfected and a variety of transcription factors have been characterized from these cells. HepG2 cells, derived from human hepatocellular carcinoma, were chosen for use because they express the CAI1 gene as was demonstrated by Northern blotting (not shown). NIH-3T3 cells were maintained in Dulbecco's modified Eagles' medium + 10% fetal calf serum and HepG2 cells were maintained in Dulbecco's modified Eagle's medium + 10% fetal calf serum.

On the day prior to transfection, the cells were removed from plates by trypsinization, seeded at 5 x 10^4 cells/10-cm plate, and grown overnight at 37°C. The cells were transfected using the calcium phosphate precipitation method of Chen and Okayama (17). Plasmid DNA (15-25 μg), prepared by cesium chloride centrifugation, was mixed with 0.5 ml of 0.25 M CaCl₂ and 0.5 ml of 50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄. The mixture was incubated at 25°C for 10-20 min and the calcium phosphate/DNA mixture was added to the medium in the plate dropwise as the plates were swirled. The plates were incubated overnight at 35°C in a 3% CO₂ atmosphere. The medium was removed and the cells were washed twice with calcium-free phosphate-buffered saline, refed, and incubated in a 5% CO₂ atmosphere at 37°C overnight.

Prior to harvesting each plate of cells was washed with phosphate-buffered saline five times and aspirated to dryness. One milliliter of 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM NaCl was added to each plate and the cells were removed with gentle cell scrapping. The cells were pelleted by centrifugation, resuspended in 150 μl of 0.25 M Tris-HCl, pH 8.0, subjected to three freeze thaw cycles and again centrifuged. The supernatant was saved at −70°C for CAT assays.

Forskolin and TPA Stimulation

For these studies NIH-3T3 cells and HepG2 cells were transfected with the appropriate CAII-pCAT basic plasmids (Fig. 1). On the day following transfection the cells were placed in 0.5% serum (as appropriate for the cell type). Twenty-four hours later the cells were stimulated with 10⁻⁵ M forskolin or 10⁻⁴ M TPA (phorbol 12-myristate 13-acetate) and harvested after 12 h. Supernatant was obtained as described above and used for chloramphenicol acetyltransferase (CAT) assay.

**CAT Assays**

CAT assays were done by a modification of the method of Seed and Sheen (18). Cell extract (5-50 μl) was added to 0.25 mM Tris-HCl, pH 8.0, final volume 110 μl, followed by 100 μM [³H]chloramphenicol (0.2 μCi, 50 μCi/mmol) and 250 μM N-butyryl-coenzyme A and incubated overnight at 37°C. In preliminary experiments I varied the incubation period from 4 to 12 h and found no difference in relative enzyme activity. The reaction mixture was extracted twice with 2 volumes of mixed xylene. After centrifugation, the organic phase containing the butyrylated [³H]chloramphenicol was removed to a scintillation vial and counted. For each experiment a standard curve was established using purified CAT enzyme (Promega) in a range of 0.001-1.0 units.

Data from the CAT assays are expressed as the mean ± S.E. of three independent transfection experiments done in quadruplicate. Each group of transfections contained either the pCAT enhancer + PstI-PstI or pCAT basic + PstI-PstI as the appropriate control. Values are compared to the appropriate control using the Student's t test. p Values ≤ 0.05 were considered significant.

**Plasmid Construction**

**CAT Plasmids**—For CAT assays I cloned the PstI-PstI fragment, truncated or mutant constructs (described below), into the pCAT plasmids (Promega) as shown in Fig. 1. I used the SV40 promoter-enhancer plasmid to establish optimum transfection conditions for the NIH-3T3 cells and HepG2 cells. The pCAT enhancer plasmid containing the SV40 enhancer was used to determine promoter activ-
CAII Gene Promoter

FIG. 2. Truncated fragments made from the 250-bp Pstl-PstI fragment of the mouse CAII gene. The Bal31--84 deletion has 84 bp deleted from the center core of the Pstl-PstI fragment. The cis regulatory elements are shown in their relative spatial arrangements.

ity. I cloned the Pstl-PstI fragment into the pCAT enhancer plasmid in the 5'--3' and 3'--5' orientations to assure the orientation specificity of promoter activity from the fragment and to establish that no aberrant transcriptional start sites were contained in the construct. The pCAT promoter plasmid containing the SV40 promoter was used to test for enhancer activity in the CAII fragment. The pCAT basic plasmid, devoid of any universal promoter or enhancer, containing CAII promoter fragments was used to test for TPA or forskolin responsiveness.

Site-directed Mutants—Site-directed mutations in the Pstl-PstI fragment were constructed in the M13 mp19 plasmid using the method of Kunkel (19). The constructs were sequenced and DNA containing the desired mutation was subcloned into the appropriate pCAT vector.

The cis regulatory sites in the CAII promoter were converted using the sequences shown in Table I.

Truncated Constructs

Using conveniently located NarI, Apal, and StuI restriction sites, I cloned the truncated fragments called Narl-PstI (216 bp), Apal-PstI (180 bp), the 160-bp fragment called the TATA domain, and the 90-bp fragment called the Spl domain (Fig. 2) into pCAT vectors. Likewise I cut the pGEM-Pstl-PstI construct at the single Stul restriction site and used Bal31 digestion to remove base pairs within the core of the Pstl-PstI fragment. I selected to use a coaestraact (Bal31--84) with 84 bp deleted between the Spl and TATA box to determine if approximation of the silencer and TATA box altered transcription (Fig. 2). The construct contained base pairs --302 minus --246 at its 5' end and base pairs --162 minus --52 at its 3' end. I used the polymerase chain reaction to generate the additional CAII promoter fragments shown in Fig. 2. I confirmed the sequence of all these fragments and cloned them into the pCAT basic and pCAT enhancer plasmids. pCRIII is identical to the 3' sequence of Bal31--84.

Band Shift Assays

To confirm the presence of a cognitive trans factor, which I refer to as CAII-CREBP, for the CRE(5'-ApZ-LE) on the mouse CAII promoter, I used band shift assays. Nuclear proteins were extracted by a modification of Hatamochi et al. (20, 21) from NIH-3T3 cells in the basal state and following stimulation with forskolin 10^-5 M at 12 and 1 h prior to harvesting.

We synthesized the oligonucleotides shown below to use in the band shift assays.

CRE (5'-Ap2-LE) 5'-ACCTCCACGGTCTCCTCCCCTTGCTCAGGTCC-3' 5'-Ap2-LE

Mutant CRE 5'-ACCTCCACGGCTCTCTCCCCTTGCTCAGGTCC-3' 3'-Ap2-LE

3'-Ap2-LE 5'-AGGTCCACTCGGTCTCTCTCCTGGGCCGCCCA-3' 3'-Ap2-LE

Mutant 3'-Ap2-LE 5'-AGGTCCACTCGGTCTCTGCTCAAGGCCCGCCA-3'

(Eq. 3)

These oligonucleotides were annealed to their complementary fragment and the 3' recessed ends were radiolabeled with Klenow fragment DNA polymerase. The binding reactions were carried out in 50
were soaked in 5% non-fat dry milk, 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (TNE-50) for 2 h at room temperature. The filters were then washed in TNE-50 and exposed to x-ray film. The proteins were transferred from the gel to nitrocellulose by electroblotting (500 mA) overnight. The nitrocellulose filters were then run over a 3% stacking, 7.5% resolving SDS-polyacrylamide gel in 0.5 mM Tris, pH 6.8, 192 mM glycine buffer. A prestained high molecular weight marker (GIBCO-Bethesda Research Laboratories) was also run. The reactions were run over a 5% polyacrylamide gel in 0.5 x TBE (25 mM Tris borate, 25 mM boric acid, 2 mM EDTA) buffer. In separate experiments, competitive binding assays were done utilizing increasing molar concentrations of nonradiolabeled CRE (5'-Ap2-LE), the 3'-Ap2-LE, the mutant oligonucleotides, or the nonspecific 30-mer as shown below.

30-mer: 5'-CCGTCCATGGCCTTCTAGAGCATGCGACCGG-3' (Eq. 4)

Southwestern Blots

Fifty micrograms of nuclear protein from NIH-3T3 cells, stimulated and unstimulated, were added to an equal volume of 0.1 M Tris Cl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M dithiothreitol, 0.001% bromphenol blue, and heated to 65 °C for 15 min. The proteins were then run over a 5% stacking, 7.5% resolving SDS-polyacrylamide gel in 25 mM Tris, pH 8.3, 192 mM glycine buffer. A prestained high molecular weight marker (GIBCO-Bethesda Research Laboratories) was also run. The proteins were transferred from the gel to nitrocellulose by electroblotting (500 mA) overnight. The nitrocellulose filters were soaked in 5% non-fat dry milk, 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (TNE-50) for 2 h at room temperature. The filters were then washed in TNE-50 and hybridized in TNE-50 plus 106 cpm/ml of the radiolabeled CRE or 3'-Ap2-LE probes. The filters were then washed at room temperature for 1 h in TNE-50 and exposed to x-ray film.

RESULTS

Sequence

We confirmed the sequence of the 250-bp fragment of the CAII gene as previously published by Venta et al. (2), however, I identified a 2-bp discrepancy in the region surrounding the Sp1 site. My sequence shows conservation of the Sp1 site but no adjacent TATA box. Thus the sequence reads CCCCGCCCCAAGCAA as compared to the published sequence CCCCGCCCCCTATAGCAA. I speculate that this 2-bp difference is due to an artifact in the chemical degradation method used by Venta et al. (2).

CAT Assays

Basal Transcription—The CAT data showed that the PstI-PstI fragment had no enhancer activity in the pCAT promoter construct (Fig. 3). In fact, the PstI-PstI fragment diminished SV40 promoter activity in NIH-3T3 cells (Fig. 3). On the other hand, the PstI-PstI fragment was a promoter in both the NIH-3T3 cells and the HepG2 cells. In the 3'-5' orientation the PstI-PstI fragment had no transcriptional activity confirming orientation specificity and indicating that aberrant start sites within the fragment were unlikely.

Analysis of the truncated fragments revealed that the Sp1 domain, PCRI, and PCRII constructs (Fig. 2) had promoter activity ≥ to the PstI-PstI fragment (Fig. 4, A and B). When tested in both cell types the TATA domain generated more CAT activity than the entire 250-bp PstI-PstI fragment. Therefore the silencer activity, identified above, could be localized to the Sp1 domain (Fig. 4, A and B). In later experiments, to identify the region of the Sp1 fragment containing the silencer, I transfected NIH-3T3 cells with the pCAT enhancer plasmid containing the NarI-PstI, ApaI-PstI, or TATA domain fragments. CAT activity from these fragments was identical (Fig. 4A). Thus, the silencer lies between bases -302 to -262 and includes the Sp1 site but no other previously identified cis regulatory region(s). Furthermore, as indicated by the CAT activity generated from the Bal31-84 construct, approximation of the silencing region, base pairs -302 to -262, to the TATA box completely abolished transcription (Fig. 4).

Although promoter activity equal to that generated by the PstI-PstI fragment could be maintained by the elements con-
A. NIH-3T3

SITE DIRECTED MUTATIONS

- WILD TYPE
- TATA
- 2Ap2-LE
- NON-CONSENSUS
  - Sp1
  - Zn
- 2Ap2-LE/TATA
- Sp1/Zn
- Sp1/Zn/2Ap2-LE

TRUNCATED FRAGMENTS

- Sp1 D
- TATA D
- TATA/TATA D
- PCR I
- PCR II
- Bal 31 Deletion

B. Hep-G2

SITE DIRECTED MUTATIONS

- WILD TYPE
- TATA
- 2Ap2-LE
- Sp1
- Zn

TRUNCATED FRAGMENTS

- TATA D

FIG. 4. Relative CAT activity from CAII-pCAT enhancer constructs. Panel A, values obtained from NIH-3T3 cells. Striped bars indicate values obtained from additional transfections (n ≥ 3). Panel B, values obtained from HepG2 cells. The values (mean ± S.E.) in each panel were obtained from n ≥ 3 separate transfections done in quadruplicate. The CAT values are expressed as a percentage of CAT activity derived from the pCAT enhancer + PstI-PstI construct in each cell type. The values in the striped bars are expressed as a percentage of CAT activity derived from the pCAT enhancer + TATA domain. *, p < 0.05.

tained on the PCRII fragment, I reasoned that studying the interactions between the consensus cis regulatory sites on the larger PstI-PstI fragment would provide more information about in vivo regulation. In order to conserve the spacial arrangement between the elements, I used pCAT constructs containing the site-directed mutations described under “Materials and Methods.”

Site-directed mutations in the TATA box or in the 2-Ap2-LE’s diminished promoter activity from the 250-bp fragment in NIH-3T3 and HepG2 cells. Likewise mutating the TATA
Fig. 5. CAT activity from CAII-pCAT basic constructs in unstimulated and forskolin (10^{-7} M) stimulated NIH-3T3 cells. Values (mean ± S.E.) are expressed as a percentage of the CAT activity derived from each construct in unstimulated cells. n ≥ 3 separate transfections done in quadruplicate. *, p < 0.05.

Fig. 6. Gel retardation analysis of nuclear protein extracts from NIH-3T3 cells in the basal state and following stimulation with forskolin (10^{-7} M). Binding reactions were conducted using the radiolabeled CRE oligonucleotide (2 × 10^{5} cpm, 0.2 ng), poly(dI-dC) (100 μg/ml), and nuclear extracts from unstimulated and stimulated cells. Lanes 1 and 2, probe with 6 and 12 μg of nuclear extract, respectively, from unstimulated cells; lanes 3 and 4, probe with 6 and 12 μg of nuclear extract, respectively, from forskolin-stimulated cells.

box on the TATA domain (TATA/TATA D) reduced CAT activity (Fig. 4A). The combination of the 2-Ap2-LE and TATA mutations (2-Ap2-LE/TATA) reduced CAT activity from the PstI-PstI fragment below levels derived from the 2-Ap2-LE or TATA mutations alone. Altering the GC content of the Sp1 and/or the GC-rich Zn finger site or the nonconsensus Sp1 site did not alter promoter activity when the TATA box and 2-Ap2-LE’s elements were functional (Fig. 4A). On the other hand, mutation of the Sp1 and Zn sites in conjunction with the 2-Ap2-LE’s (Sp1/Zn/2-Ap2-LE) diminished CAT activity below that of the 2-Ap2-LE mutation (Fig. 4A).

TPA and Forskolin Stimulation—To determine if the 2-Ap2-LE’s were functional CRE or protein kinase C-responsive elements, I stimulated NIH-3T3 and HepG2 cells containing the CAII-pCAT basic constructs with 10^{-6} M forskolin or 10^{-8} M TPA (Fig. 5). There was no increase in CAT activity following TPA administration. In additional experiments I conducted a detailed time course for TPA stimulation extending from 1 to 24 h but found no increase in CAT activity. In similar experiments, forskolin (10^{-5} M) did not increase CAT activity from the pCAT basic PstI-PstI construct in HepG2.
cells. However, forskolin enhanced CAT activity in the NIH-3T3 cells directed from the wild type (PstI-PstI fragment), TATA mutant, the 2-Ap2-LE mutant, TATA domain, and the PCRII fragment (Fig. 5). As shown in Fig. 5, the response to forskolin was blunted by mutation in the Sp1 and Zn finger sites and there was no CRE on the Sp1 domain or the PCRII construct.

**Band Shift Assays**—Band shift assays indicated that nuclear protein from forskolin stimulated and unstimulated NIH-3T3 cells bound equally well to the 5'-Ap2-LE(CRE) (Fig. 6). Three distinct DNA/protein species were identified and each was competitively inhibited by the CRE and the 3'-Ap2-LE but not the 30-mer (non-specific), respectively, with extract; lanes 4 and 5, probe plus 50 and 100 M excess unlabeled 3'-Ap2-LE with extract. Lanes 6 and 7, probe plus 50 and 100 M excess unlabeled CRE with extract. Lane 8, 3'-Ap2-LE probe alone.

**Southwestern Blots**—Results from Southwestern blots employing the 5'-Ap2-LE and extracts from NIH-3T3 cells show that CAII-CREBP is a single protein of about 65 kDa (Fig. 10A). The size of CAII-CREBP is identical in forskolin-stimulated and unstimulated cells. Southwestern blotting of the same extracts using the 3'-Ap2-LE probe revealed the CAII-CREBP band and a second larger band of 100 kDa in size (Fig. 10B). The binding of the larger protein to the 3'-Ap2-LE oligonucleotide may account for the difference in the band shift profiles between the 3'-Ap2-LE and the CRE.

**DISCUSSION**

In the present study I have identified that the PstI-PstI fragment of the mouse CAII gene contains promoter function and defined cis regulatory regions on this fragment that are essential for basal and cAMP-stimulated transcription. The 250-bp PstI-PstI fragment so efficiently directs CAT transcription with the SV40 enhancer in HepG2 cells that its activity is greater than the SV40 promoter (Fig. 3). The PstI-PstI fragment was also an efficient promoter in NIH-3T3 cells that do not express the CAII gene at a level detectable by Northern blotting. Thus, it is apparent that cellular specificity for CAII gene expression is not determined by elements located on the PstI-PstI fragment.

Further analysis of CAT activity from truncations of the PstI-PstI fragment revealed that the Sp1 domain can function as a “TATA-less” promoter and that base pairs –302 to –262 on this fragment contain silencer activity. Silencer strength does not appear to depend on the GC content of the Sp1 site since mutation of the site does not increase CAT activity from the PstI-PstI fragment (Fig. 4). The elements located on the TATA domain maintain maximal promoter activity while those on PCRII supply promoter function equal to the 250-bp PstI-PstI fragment. The 5'-region of the TATA domain from base pairs –212 to –187 is critical for optimal promoter function; however, this region contains no known cis regulatory element(s). It is possible that an as yet unidentified cis element lies here or that this region anchors binding of trans factors to the 3'-region of the TATA domain.

The TATA box is the most critical element directing transcription from both the PstI-PstI fragment and the TATA domain. Mutation of the TATA box reduced promoter activity to 20 and 50% of control in NIH-3T3 and HepG2 cells, respectively (Fig. 4, A and B). This residual promoter activity may be explained by the characteristics of the TATA-binding protein, transcription factor IID, which protects several base pairs surrounding the TATA box and complexes with other proteins to form a unit that directs transcription (23). Thus the “TTTTT” mutation in the four consensus TATA base pairs may alter protein binding affinity and decrease but not eliminate transcriptional activity. On the other hand, the remaining cis regulatory sites on the PstI-PstI fragment may sustain the residual promoter function. In either case disruption of the TATA sequence has more profound effects on transcription directed by the CAII promoter in the NIH-3T3 cells than the HepG2 cells.

The consensus and nonconsensus Sp1 sites and the Zn finger site on the PstI-PstI fragment do not function to enhance transcription driven by the TATA box. Yet, the GC-rich Sp1 domain containing the Sp1 and Zn finger sites can act as an efficient TATA-less promoter in both cell types (Fig. 4, A and B). Whether this action is employed in vivo remains unknown. By measuring CAT activity derived from the Sp1/Zn/2-Ap2-LE and 2-Ap2-LE mutants I have shown that the Sp1 and Zn finger sites do enhance transcription from the TATA box when the Ap2-LEs are inactivated. Although they are apparently inactive when NIH-3T3 cells are in the basal state, the Sp1 and Zn finger elements enhance the transcriptional response to forskolin generated from the PstI-PstI fragment (Fig. 5). Thus, these GC-rich sites may act in conjunction with other cis and trans factors to regulate cAMP-mediated transcription.

In contrast to the Sp1 and TATA proteins that are well
Figure 9. Gel retardation analysis of nuclear protein extracts from forskolin-stimulated NIH-3T3 cells with the mutant CRE and mutant 3′-Ap2-LE probes. A, lane 1, CRE probe with 6 μg of extract; lanes 2–4, mutant CRE probe with 6, 12, 24 μg of extract, respectively. B, lane 1, CRE probe and 6 μg of extract; lanes 2 and 3, CRE probe and 6 μg of extract with 50 and 100 m excess mutant CRE-unlabeled oligonucleotide, respectively. C, lane 1, 3′-Ap2-LE probe with 6 μg of extract; lane 2, mutant 3′-Ap2-LE probe with 24 μg of extract.

Figure 10. Southwestern blotting of nuclear protein extracts from forskolin-stimulated nonstimulated NIH-3T3 cells with the CRE or 3′-Ap2-LE probe. A, lane 1, CRE/protein binding from nonstimulated cells; lane 2, CRE/protein binding from forskolin-stimulated cells. B, 3′-Ap2-LE/protein binding from stimulated cells. Prestained protein marker sizes are shown to the right of each panel.

characterized, the function and cognitive protein for the 2-Ap2-LEs contained on the PstI-PstI fragment have not been previously described. Mutations in both the Ap2-LEs diminish PstI-PstI promoter function but CAT activity from the truncated fragments demonstrates that only the 3′-Ap2-LE is necessary for promoter activity. On the other hand, the 5′-Ap2-LE is required for CRE activity. Oddly, mutations in the 2-Ap2-LEs that decrease promoter activity and inhibit nuclear protein binding do not abolish the response to forskolin from the PstI-PstI fragment. But as noted above, the Sp1 and Zn finger sites potentiate the response to forskolin and I reason that protein kinase A-mediated modifications in these other trans factors may contribute to conserve cAMP responsiveness from the 2-Ap2-LE mutation made in the PstI-PstI fragment. As such the inter-relationships between multiple cis-trans interactions would govern the cAMP responsiveness of the complex PstI-PstI fragment. Although multiple cis factors on the PstI-PstI fragment contribute to forskolin responsive transcription in NIH-3T3 cells, these factors are not sufficient to mediate a similar response in HepG2 cells. Thus, the modulation of CAII transcription requires cell-specific factors and hepatic factors may not be included on the PstI-PstI fragment.

We have confirmed that the Ap2-LEs can function as cAMP-responsive elements and basal promoter elements. Both elements bind a cognitive protein of ~65 kDa in size, CAII-CREBP. Although I have not completed the DNase footprinting of the bases required for binding, the results from our band shift assays employing the 5′- and 3′-Ap2-LEs and their mutated constructs suggest that the CCTCCCT se-
quence, which is common to both the Ap2-LEs, is the critical region for CAII-CREBP binding. Although multiple DNA-protein complexes are seen in our band shift assays, they are all competitively inhibited by the presence of the CRE. In the same light, Southwestern blots show that only CAII-CREBP is needed for Ap2-LE binding. Although CAII-CREBP may homo- or heterodimerize, its binding to the Ap2-LEs is not dependent on protein-protein interactions. The 3'-Ap2-LE oligonucleotide binds to a second protein of ~100 kDa in size and I speculate that the GC-rich portion of this oligonucleotide probe, which contains the nonconsensus Sp1 site, may be binding to the Sp1 protein that is 100 kDa. Nuclear protein binding to the 3'-Ap2-LE is completely inhibited by the CRE which does not contain the GC-rich sequence and I speculate that the conditions of our binding assay may not be ideal for a zinc finger protein, such as Sp1, to bind to this sequence. I am presently working to confirm this hypotheses. The size of CAII-CREBP (~65 kDa) is larger than the 52-kDa Ap2 protein confirming that the Ap2-LEs do not bind Ap2 (12, 24).

In conclusion, I have documented that the 3'-Ap2-LEs on the CAI1 gene affect basal transcription, that the 5'-Ap2-LE is required for cAMP-mediated increases in transcription, and that both elements bind a nuclear protein (CAI1-CREBP) of ~65 kDa in size. I plan to pursue identification of this protein and to determine if Ap2-LE cis regulatory sites are involved in the regulation of other genes.

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22. Deleted in proof