Journal of Cellular Biochemistry 57:711–723 (1995)

Protein That Binds to the Distal, but Not to the Proximal, CCAAT of the Human Thymidine Kinase Gene Promoter

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Abstract Mobility shift assays were used to examine protein binding to the human TK gene CCAAT boxes. Similar protein binding patterns were observed with probes containing either the proximal or distal CCAAT. However, probes containing both CCAAT boxes in which one of the CCAAT boxes was inactivated by mutation did not demonstrate identical binding patterns. One of the complexes formed with the longer probes was only observed when the distal CCAAT was intact. This species was not formed with probes that only contained an intact proximal CCAAT, and its formation could only be competed by oligonucleotides containing the distal CCAAT motif. This observation reveals the existence of a protein that can bind to the distal, but not to the proximal, CCAAT of the human TK promoter. This protein may account for the previous observation that the two CCAAT motifs are not functionally equivalent. The protein that binds to the distal, but not to the proximal, CCAAT (DTK-CBP) was also present in two human cell lines. Significantly more DTK-CBP was present in nuclear extracts of HepG2 and WI38 cells than in TK$^-$ts13 cells. However, this protein was not observed in three different murine cell lines and one primary culture. Its abundance in some human cell lines suggests it might modulate the expression of human TK mRNA in cells that express this protein. © 1995 Wiley-Liss, Inc.

Key words: mobility shift, transcription, DNA-binding protein, HepG2, WI38, TK$^-$ts13

Thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) is a salvage enzyme that is coordinately expressed with enzymes that synthesize DNA. The amount of TK enzyme activity increases at the boundary between G_1 and S phases of the cell cycle, and remains elevated throughout S phase [Brent et al., 1965; Littlefield, 1966; Stubblefield and Muller, 1965; Liu et al., 1985]. The amount of TK mRNA also increases at the onset of S phase, when quiescent cells are stimulated to proliferate [Liu et al., 1985; Stuart et al., 1985; Coppock and Pardee, 1987]. Therefore, TK has been used as a model for cell cycle-regulated expression of G_1/S phase boundary genes. The regulation of TK expression has been demonstrated to be very complex. Transcriptional as well as several post-transcriptional mechanisms have been show to participate in the cell cycle regulation of TK [Coppock and Pardee, 1987; Stewart et al., 1987; Kim et al., 1988; Travali et al., 1988; Gudas et al., 1988; Sherley and Kelly, 1988; Gross and Merrill, 1988, 1989; Lipson and Baserga, 1989; Chang, 1990; Ito and Conrad, 1990; Kauffman and Kelly, 1991; Kauffman et al., 1991).

Binding sites for SP1 and CCAAT boxes have been demonstrated to be important determinants of the amount of TK mRNA produced [Kreidberg and Kelly, 1986; Lipson et al., 1989; Arcot et al., 1989; Mao et al., 1995]. Recently, the CCAAT binding protein NF-Y has been demonstrated to be the protein that binds to the CCAAT boxes in the human TK promoter [Chang and Liu, 1994]. Footprinting assays have suggested that protein binding to the proximal CCAAT precludes binding to the distal CCAAT [Arcot and Deininger, 1992]. This conclusion was supported by the observation that increasing the distance between the two CCAAT boxes to reduce steric hindrance increased the strength

Received August 19, 1994; accepted September 23, 1994.

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of the TK promoter [Arcot and Deininger, 1992]. However, data from experiments in which one of the CCAAT boxes was mutated suggest that the presence of the distal, as well as the proximal, CCAAT box is necessary for full promoter strength [Mao et al., 1995]. Therefore, oligonucleotide probes spanning the two CCAAT boxes were synthesized by PCR (Table I) and used to examine CCAAT-binding protein interactions with the mobility shift assay [Fried and Crothers, 1981; Garner and Revzin, 1981; Gilman et al., 1986]. The results suggest that proteins can bind to both CCAAT boxes simultaneously. In addition, a rare protein that binds exclusively to the distal CCAAT element was identified. This protein was more abundant in two human cell lines than the hamster cell line in which it was originally observed. However, its presence could not be readily detected in several types of murine cells.

MATERIALS AND METHODS

Cell Lines

TK⁻ts13 Syrian hamster fibroblasts [Shen et al., 1982], a TK-deficient cell line derived from ts13 cells [Talavera and Basilico, 1977], were grown at 34°C in DMEM supplemented with

TABLE I. Mobility Shift Probes and Competitors*

Probe	Sequence
PTK:	GCGGGGCCGGCTCGTGATTGGC- CAGCACGCCGT CGCCCCGGCCGAGCACTAACCGGT CGTGCGGCA
DTK:	CAGCGGCCGGGCGCTGATTGGC- CCCATGGCGGC GTCGCCGGCCCGCGACTAACCGGG GTACCGCCG
TKIC:	TGGCCCCATGGCGGCGGGGCCG- GCTCGTGA CCGGGGTACCGCCGCC CCGGCC- GAGCACTA
PTKM1:	GCGGGGCCGGCTCGTGATCAGC- CAGCACGCCGT CGCCCCGGCCGAGCACTAGTCGGT CGTGCGGCA
MHC:	CGTAAACATTTTTCTGATTGGT- TAAAAGTTGAG GCATTTGTAAAAAGACTAAC- CAATTTTCAACTC
HSV:	CCCAGCGTCTTGTCATTGGC- GAATTCGAACA GGGTCGCAGAACAGTAACCGCT- TAAGCTTGT
αG:	GGCGGCGCTCATTGGCTGGCGCG- GAGCCCG CCGCCGCGAGTAACCGACCGC- GCCTCGGGC
βG:	GTGTGAGCAGATTGGCCCTTAC- CAGGGTGT CACACTCGTCTAACCGGGAATGGT CCCACA
NF1:	ATTTTGGCTTGAAGCCAATATG ATAAAACCGAACTTCGGTTATA
AP3:	CTAGTGGGACTTTCCACAGATC GATCACCCTGAAAGGTGTCTAG
SP1:	GATCGATCGGGGCGGGGCGATC CTAGCTAGCCCCGCCCCGCTAG
EGR1:	CGCCCTCGCCCCCGCGCCGG GCGGGAGCGGGGGCGCGGCC
AP2:	TAGTCGACCCCAGGCCATGGTA ATCAGCTGGGGTCCGGTACCAT
TK2C:	CAGCGGCCGGGCGCTGATTGGC- CCCATGGCGGCGGGGCCGGCTC- GTGATTGGCCAGCACGCCGT GTCGCCGGCCCGCGACTAACCGGG GTACCGCCGCCCCGGCCGAGCAC- TAACCGGTCGTGCGGCA
TK2CPM1:	CAGCGGCCGGGCGCTGATTGGC- CCCATGGCGGCGGGGCCGGCTC- GTGATCAGCCAGCACGCCGT GTCGCCGGCCCGCGACTAACCGGG GTACCGCCGCCCCGGCCGAGCAC- TAGTCGGTCGTGCGGCA
TK2CDM1:	CAGCGGCCGGGCGCTGATCAGC- CCCATGGCGGCGGGGCCGGCT CGTGATTGGCCAGCACGCCGT GTCGCCGGCCCGCGACTAGTC- GGGGTACCGCCGCCCCGGCCGA GCACTAACCGGTCGTGCGGCA
TK2CPDM1:	CAGCGGCCGGGCGCTGATCAGC- CCCATGGCGGCGGGGCCGGCT CGTGATCAGCCAGCACGCCGT GTCGCCGGCCCGCGACTAGTC- GGGGTACCGCCGCCCCGGCCGA GCACTAGTCGGTCGTGCGGCA

*Competitors: PTK, proximal human TK CCAAT; DTK, distal human TK CCAAT; TKIC, contains the sequence between the two human TK CCAAT boxes; PTKM1, PTK probe containing a mutated CCAAT; MHC, human MHC class II Eα Y-box CCAAT; HSV, Herpes simplex virus CCAAT; αG, mouse α-globin CCAAT; βG, mouse β-globin CCAAT; NF1, nuclear factor 1/CTF binding site; AP3, AP3 binding site (TGTGGAAAG); SP1, SP1 binding site (GGGCGG); EGR1, EGR1 binding site (GCGGGGGCG); AP2, AP2 binding site (CCCCAGGC); TK2C, proximal and distal TK CCAAT boxes; TK2CPM1, TK2C with the proximal CCAAT mutated to CTGAT; TK2CDM1, TK2C with the distal CCAAT mutated to CTGAT; TK2CPDM1, TK2C, with both CCAAT boxes mutated to CTGAT.

10% calf serum. The cells were made quiescent by a combination of contact inhibition and serum deprivation (0.1%) for 48 h prior to stimulation.

BALB/c 3T3 cells [Aaronson and Todaro, 1968] were grown at 37°C in DMEM supplemented with 5% fetal bovine serum and 5% calf serum. WI38 cells [Hayflick and Moorhead, 1961] were grown at 37°C in MEM with Earle's salts, which was supplemented with 10% fetal bovine serum and nonessential amino acids. HepG2 cells [Aden et al., 1979] were grown at 37°C in MEM with Earle's salts, which was supplemented with 10% fetal bovine serum, nonessential amino acids, basal minimum Eagle's vitamins, and sodium pyruvate. Swiss 3T3 cells [Todaro and Green, 1963] were grown in DMEM supplemented with 10% fetal bovine serum. Primary mouse splenocytes were isolated as previously described [Gai et al., 1992] and maintained in DMEM supplemented with 10% fetal bovine serum. The murine, clonal T lymphocyte cell line, L2 were grown in DMEM supplemented with 10% fetal bovine serum and 400 units of IL-2 on a feeder layer of irradiated splenocytes [Shipman et al., 1988]. Mouse splenocytes and L2 cells were stimulated with concanavalin A or IL-2, respectively, for 48 h prior to isolation of nuclear extracts [Gai et al., 1992].

Mobility Shift Assays

Oligonucleotides containing single protein binding sites that were used for probes or competitors in mobility shift assays [Fried and Crothers, 1981; Garner and Revzin, 1981; Gilman et al., 1986] (Table I) were synthesized on an Applied Biosystems Model 391 EP Oligonucleotide Synthesizer using phosphoramidite chemistry. Probes containing two CCAAT boxes were prepared by PCR using the coding strand oligonucleotide from the DTK probe/competitor and noncoding strand oligonucleotide from the PTK probe/competitor as primers, and Eco RI digested plasmids containing intact or mutated CCAAT boxes [Mao et al., 1995] as a template. Probes and PCR amplification primers were end labeled with [γ-^{32}P]ATP and polynucleotide kinase. Prior to use, probes and competitors were purified on polyacrylamide gels.

Nuclear proteins were extracted by the method of Dignam et al. [1983] and quantitated by the method of Bradford [1976], using ovalbumin as a standard. Five micrograms of nuclear extract was added to a binding mixture consisting of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 3 μg poly-(dIdC), and the indicated amount of competitor. The binding reaction was initiated by the addition of 5,000 cpm of probe. After 30 min, 2 μl of 0.02% bromophenol blue was added and the mixture was fractionated on a 4% polyacrylamide gel in an electrophoresis buffer consisting of 190 mM glycine, 1 mM EDTA, and 25 mM Tris, at a constant current of 30 mA. The dried gels were exposed to Kodak XAR-5 film for autoradiography.

RESULTS

Mobility Shift Assays With Probes Containing a TK CCAAT Motif

Mobility shift experiments were initiated to visualize protein binding to the proximal and distal CCAAT boxes (Fig. 1). Two predominant protein complexes were observed with both the PTK probe (Fig. 1A) and the DTK probe (Fig. 1B). In addition to these two predominant complexes, three less abundant complexes were observed with the PTK probe, while five less abundant complexes could be observed with the DTK probe. The smaller of the less abundant complexes formed with the DTK probe could only be visualized in autoradiographs that had been overexposed as shown in Figure 1B. Identical autoradiographic exposures demonstrated that less of the abundant complexes were formed with the DTK probe than with the PTK probe (not shown). For convenience, the complexes have been identified alphabetically beginning with the largest. The abundance of most of the complexes formed with the PTK probe increased when nuclear extracts from serum-stimulated cells were used, but not when the cells were stimulated with serum at the nonpermissive temperature (Fig. 1A). With the DTK probe, the abundance of most of the complexes did not increase with serum stimulation at the permissive temperature, and were significantly less abundant with serum stimulation at the nonpermissive temperature (Fig. 1B).

Competition for Protein Binding to the Proximal TK CCAAT

To characterize the various complexes further, the ability of different competitors to inhibit their formation was examined (Fig. 2). All of the complexes formed with a PTK probe were effectively competed by PTK, but not by a competitor that contained a mutant CCAAT box (PTKM1). In addition to the PTK competitor,

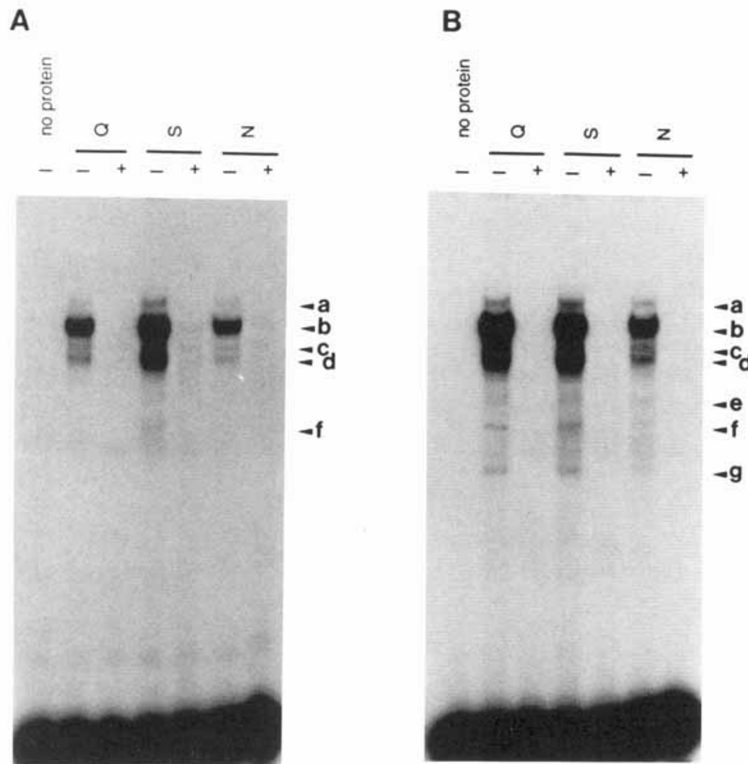

Fig. 1. Protein binding activity to the proximal and distal CCAAT boxes. Mobility shift assays were performed as described in the text, using the isolated CCAAT probes PTK (**A**) and DTK (**B**) (see Table I) and nuclear extracts from quiescent cells (Q) or cells that were stimulated with serum for 24 h at the permissive temperature of 34°C (S) or at the nonpermissive temperature of 39.6°C (N). The first lane of each panel contained probe without nuclear extract. The presence or absence of competitor (50 ng of unlabeled probe) added to the binding reactions are indicated above each lane by + or –, respectively.

MHC was very effective at inhibiting formation of the complexes. Although it is not clearly visible in the exposure shown, DTK was somewhat less effective at competing for formation of these complexes. HSV, βG, and αG were progressively less effective at inhibiting formation of the complexes, while competitors containing binding sites for SP1, NF1, or AP3 were completely ineffective. The results obtained with the DTK probe were similar (not shown).

Mobility Shift Assays With Probes Spanning Both TK CCAAT Boxes

In order to determine if proteins could bind to both CCAAT boxes simultaneously, a longer probe (TK2C) spanning the region containing both CCAAT boxes was prepared by PCR. Similar probes were prepared in which either the proximal (TK2CPM1) or distal (TK2CDM1) CCAAT was mutated, or in which both CCAAT boxes (TK2CPDM1) were mutated (Table I). Each of these probes was used in mobility shift assays with nuclear extracts from serum-deprived TK^- ts13 cells (Q) or cells that had been stimulated with serum at the permissive (S) or nonpermissive (N) temperature (Fig. 3).

Numerous complexes were formed with the TK2C probe (Fig. 3A). Five of the complexes appear to represent nonspecific protein binding, since their formation was not prevented by an excess of unlabeled competitor (shown to the right of Fig. 3B with open arrows). For convenience, the specific complexes have been labelled alphabetically beginning with the one of least electrophoretic mobility. The two smallest complexes that appeared to be specific (g and h) were also formed when TK2CPDM1 was used as a probe (Fig. 3B). Since both CCAAT boxes are mutated in TK2CPDM1, observation of these complexes indicates that the proteins responsible for formation of complexes g and h did not recognize the CCAAT motif.

Most of the complexes formed with the TK2C probe were equally abundant when nuclear extracts from serum-deprived cells, or from cells stimulated with serum at either the permissive or nonpermissive temperature, were used as a source of binding proteins. Complex f, however,

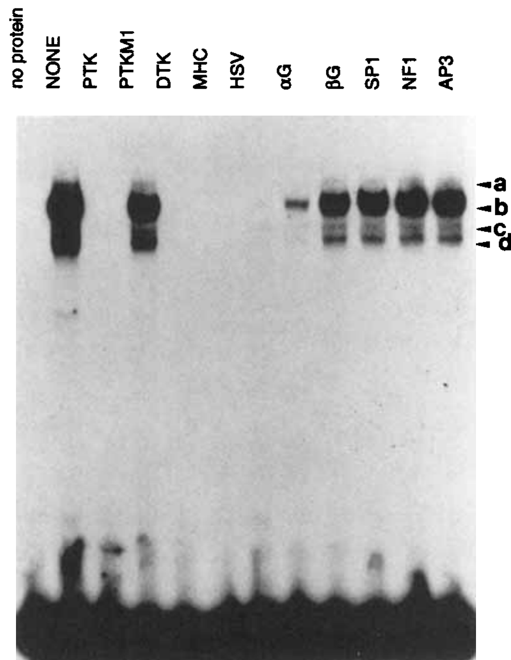

Fig. 2. Competition for protein binding to the proximal TK CCAAT box. Mobility shift experiments were performed using the proximal (PTK) CCAAT box probe and nuclear extracts from cells stimulated with serum at the permissive temperature. The identity of competing oligonucleotides (Table I) is shown above each lane.

was less abundant with nuclear extracts from cells stimulated at the nonpermissive temperature.

Mobility Shift Assays With Long Probes Containing a Mutated CCAAT

Analogs of TK2C in which either the proximal (TK2CPM1) or distal (TK2CDM1) CCAAT motif were inactivated by mutation were used as probes to ascertain if any of the complexes identified with TK2C resulted from protein binding to only one of the CCAAT boxes (Fig. 3C,D). Comparison of the complexes formed with these two probes revealed several interesting observations.

1. The four largest complexes formed with each of these probes (labeled b–e) were similar to those formed with the other. However, considerably less of each of the complexes was formed with TK2CPM1 (Fig. 3C), which contains an intact distal and a mutated proximal CCAAT. These data are consistent with a previous observation of higher affinity protein binding to the proximal than to the distal CCAAT box of the human TK promoter [Arcot et al., 1989].

2. While more of the four largest specific complexes (b–e) were formed with TK2CDM1, less of the four main nonspecific complexes were observed. These observations are probably related, since strong binding to one or more abundant proteins could deplete the concentration of free probe to below the K_d of the nonspecific complexes, thus reducing their formation.

3. Complex a, which was formed with the TK2C probe, was not formed with either TK2CPM1 or TK2CDM1. Since both of the latter probes contain only one intact CCAAT, this observation suggests that complex a represents protein binding to both CCAAT boxes simultaneously.

4. The most interesting observation, however, was the formation of complex f with the probe that contains an intact distal CCAAT (TK2CPM1, Fig. 3C), but not with the probe that contains an intact proximal CCAAT (TK2CDM1, Fig. 3D). This observation identifies a protein that binds specifically to the distal, but not the proximal, CCAAT motif in the human TK promoter. The binding activity of this protein is comparable in serum-deprived cells and in cells that were stimulated at the permissive temperature, but much lower in cells stimulated at the nonpermissive temperature.

Competition for Protein Binding to Long Probes With Mutated CCAAT Motifs

In order to more fully characterize the specificity of the various complexes formed with the probes described above, different oligonucleotides (Table I) were used as competitors in mobility shift assays (Fig. 4). TK2C complexes a–e were competed similarly by the various probes (Fig. 4A). Each was competed by any oligonucleotide that contained at least one CCAAT box, with the proximal CCAAT containing oligonucleotides (TK2C, TK2CDM1, PTK) being more effective competitors than those which contained only the distal CCAAT (TK2CPM1, DTK). The TK2CPDM1 competitor, in which both CCAAT boxes are mutated, only competed for formation of the two complexes formed by proteins that do not bind to the CCAAT boxes (g and h). The competitor containing TK promoter sequences located between the two CCAAT boxes (TKIC), and those containing binding sites for other DNA binding proteins, did not compete for formation of complexes a–e.

The pattern of competition for complex f was quite different from that of complexes a–e. The

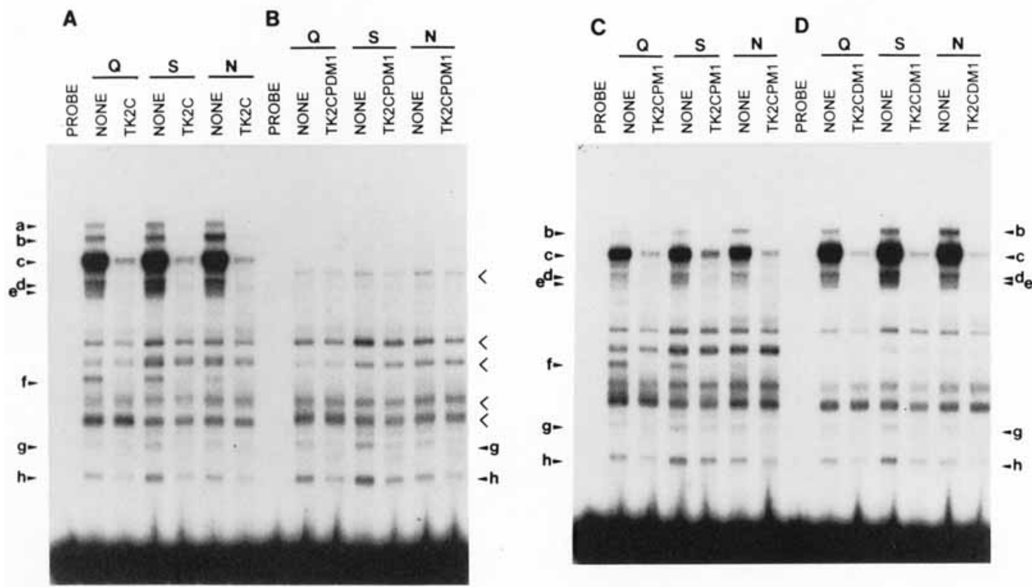

Fig. 3. Protein binding to probes containing two CCAAT boxes. A mobility shift assay was performed with the TK2C probe (A) containing two intact CCAAT boxes, the TK2CPDM1 probe (B) containing two mutated CCAAT boxes, the TK2CPM1 probe (C) containing an intact distal and a mutated proximal CCAAT box, or the TK2CDM1 probe (D) containing an intact proximal and a mutated distal CCAAT box and nuclear extracts from cells that were serum deprived (Q) or stimulated with serum at the permissive (S) or nonpermissive (N) temperatures. The presence and identity of any competitors (50 ng) that were added are indicated above each lane. Complexes that could be demonstrated to represent specific binding by the fact that their formation could be inhibited by a competitor are marked with solid arrows adjacent to each panel. Each specific complex formed with the TK2C probe was assigned a succeeding letter designation, beginning with the one with the least electrophoretic mobility. Specific complexes formed with the other probes were identified with the same designation as for the TK2C probe. Nonspecific complexes are marked with open arrows adjacent only to B.

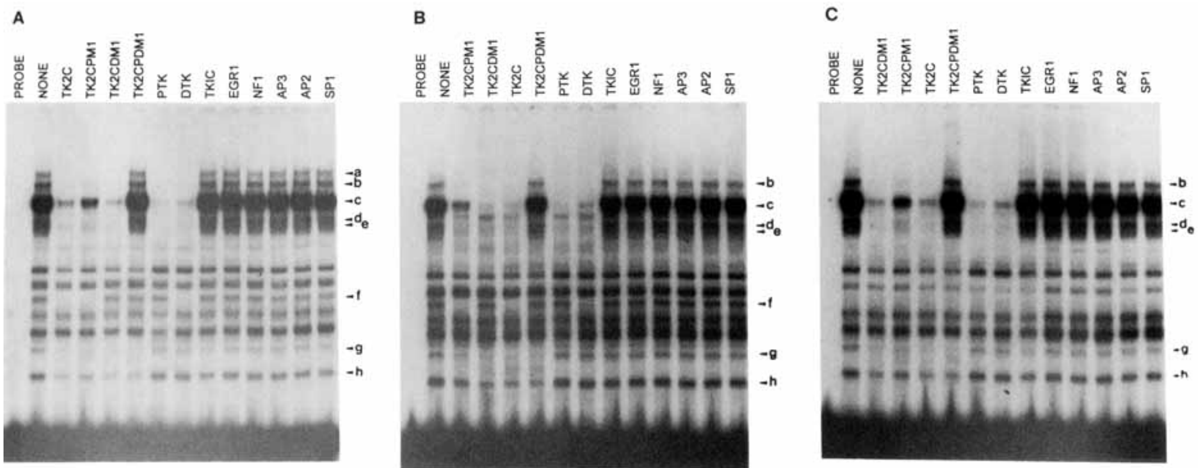

Fig. 4. Competition of complexes formed with the double CCAAT probes with heterologous competitors. A mobility shift assay was performed using TK2C (A), TK2CPM1 (B), or TK2CDM1 (C) as a probe and nuclear extracts from serum stimulated cells. Fifty nanograms of the competitor (see Table I) indicated at the top of each lane was added to the binding mixtures. The specific complexes for each probe are indicated to the right of each panel.

formation of complex f was only inhibited by TK2C, TK2CPM1, and DTK, all of which contain an intact distal CCAAT. Competitors containing only a proximal CCAAT (TK2CDM1, PTK) were unable to compete for formation of complex f.

Similar observations were made with the TK2CPM1 (Fig. 4B) and TK2CDM1 (Fig. 4C) probes. Complexes b–e (complex a was not formed) were most effectively competed by oligonucleotides containing a proximal CCAAT, but were competed by any competitor with an intact

CCAAT. Complex f was only formed with the TK2CPM1 probe, and was only competed by oligonucleotides that contain a distal CCAAT box. Competitors that contained only a proximal CCAAT did not compete for formation of complex f. Thus, these observations confirm that complex f is formed by a protein that can bind to the distal, but not to the proximal CCAAT in the human TK promoter. For the convenience of discussion, we will designate this protein as DTK-CBP until it is identified.

Mobility Shift Assays With Nuclear Extracts From Other Cell Lines

In order to identify other cell lines that might contain the protein that bound to the distal TK CCAAT (DTK-CBP), nuclear extracts were isolated from the following cell lines after 18 h of serum stimulation: BALB/c 3T3, an embryonic murine cell line [Aaronson and Todaro, 1968] that demonstrates a confluent morphology distinct from that of fibroblasts; WI38, a diploid human fibroblast cell line [Hayflick and Moorhead, 1961]; and HepG2, a minimal deviation human hepatocarcinoma cell line [Aden et al., 1979]. These nuclear extracts were used for mobility shift assays with the TK2C probe, and compared to nuclear extracts isolated from stimulated TK$^-$ts13 cells.

The abundance and electrophoretic mobility of the various complexes formed between the TK2C probe and each of the nuclear extracts was different for each cell line (Fig. 5). Of particular interest, however, was the complex formed by DTK-CBP. As was shown above, the abundance of this complex was low with nuclear extracts from TK$^-$ts13 cells. Significantly more was observed with nuclear extracts from HepG2 and WI38 cells. In contrast, none of this complex could be observed with nuclear extracts from BALB/c 3T3 cells (Fig. 5).

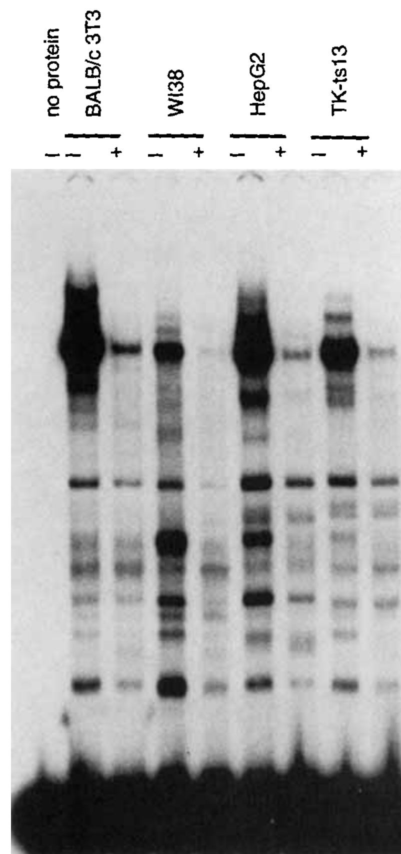

Fig. 5. Comparison of nuclear extracts from different cells. Mobility shift assays were performed with the nuclear extracts indicated above each lane, and the TK2C probe (see Table I). The presence or absence of 50 ng of TK2C competitor in the incubation mixture is indicated by + or −, respectively. Specific protein binding is defined by the ability of such an excess of competitor to prevent the formation of a complex. The complex representing the protein that binds uniquely to the distal CCAAT box (DTK-CBP) is indicated by an arrow.

Mobility Shift Assays With Long Probes Containing CCAAT Mutations and Various Nuclear Extracts

In order to confirm that the indicated complex was formed by DTK-CBP, mobility shift assays were performed with probes containing an inactivating mutation of either the proximal (TK2CPM1) or distal (TK2CDM1) CCAAT (Table I). DTK-CBP was identified by the fact that it binds to the distal but not to the proximal CCAAT. Thus, a complex with this protein was formed with nuclear extracts from TK$^-$ts13 cells and TK2C or TK2CPM1 probes, but not with the TK2CDM1 probe which only contains an intact proximal CCAAT. With nuclear extracts from both WI38 (Fig. 6B) and HepG2 (Fig. 6C) cells, a specific complex was formed with the TK2C and TK2CPM1 probes, but not with the TK2CDM1 probe. This clearly indicates that DTK-CBP is present in both of these human cell lines. In contrast, a similar complex could not be unequivocally identified with nuclear extracts from BALB/c 3T3 cells using any of the probes (Fig. 6A). Thus, DTK-CBP appears to be present in several human cell lines, but could not be readily observed in BALB/c 3T3 cells.

Competition for Protein Binding to Long Probes With Mutated CCAAT Motifs Using Nuclear Extracts From Various Cell Lines

The observations above were also confirmed by competition assays (Fig. 7). In the above

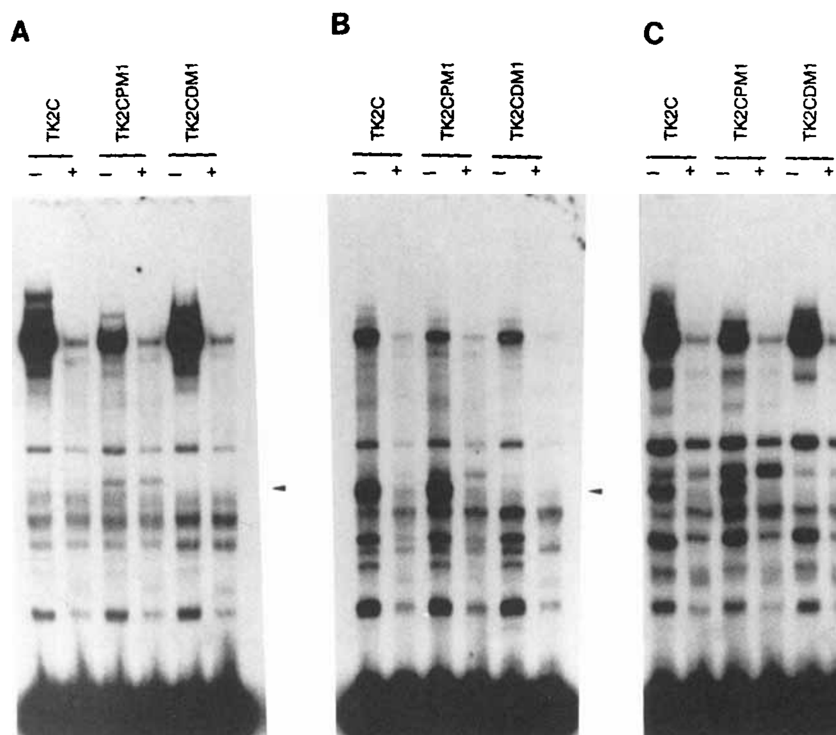

Fig. 6. Comparison of protein binding to probes with mutated CCAAT motifs. Mobility shift assays were performed with the indicated probes and nuclear extracts from BALB/c 3T3 cells (A), WI38 cells (B), or HepG2 cells (C). The presence or absence of 50 ng of unlabeled probe in the incubation mixture is indicated above each lane by + or −, respectively. Specific protein binding is defined by the ability of such an excess of competitor to prevent the formation of a complex. The DTK-CBP complex, or its expected position, is indicated by an arrow.

experiments with $TK^{-}ts13$ nuclear extracts, we demonstrated that formation of the DTK-CBP complex could be inhibited by any competitor that contained the distal CCAAT motif from the human TK promoter, but not by competitors that only contained the proximal CCAAT. Similarly, the complex formed with DTK-CBP from either WI38 (Fig. 7B) or HepG2 (Fig. 7C) nuclear extracts could be competed by an excess of TK2C, TK2CPM1, or DTK, but not by TK2CDM1, TK2CPDM1, or PTK. As observed above, a DTK-CBP complex was not observed with nuclear extracts from BALB/c 3T3 cells (Fig. 7A).

Further Examination of Protein Binding Using Nuclear Extracts From Other Murine Cells

These experiments demonstrate that DTK-CBP is present in two different human cell lines, as well as in $TK^{-}ts13$ cells, but could not be observed in BALB/c 3T3 cells. In order to determine if DTK-CBP might be more abundant in other murine cells, nuclear extracts were isolated from serum-stimulated Swiss 3T3 fibroblasts, from concanavalin A stimulated primary mouse splenocytes, and from the T cell clone, L2, after stimulation with IL-2. These nuclear extracts were used for mobility shift assays with the TK2C probe (Fig. 8). Although numerous specific complexes could be observed with each of the nuclear extracts, no complex with the electrophoretic mobility of the DTK-CBP complex was present. With nuclear extracts from Swiss 3T3 cells, two very minor complexes with a slightly lower electrophoretic mobility could be observed. However, these complexes appear to be distinct from that formed by DTK-CBP, since similar complexes were observed with nuclear extracts from BALB/c 3T3, HepG2 and $TK^{-}ts13$ cells (Fig. 5). These complexes can only be observed in overexposed autoradiographs (such as Fig. 8), and their abundance is too low to fully characterize the proteins that form them.

DISCUSSION

$TK^{-}ts13$ Cells

$TK^{-}ts13$ cells contain a temperature sensitive mutation in cell cycle progression so that they arrest in the second half of G_1 at the nonpermissive temperature of 39.6°C. When these cells are arrested at the nonpermissive temperature, im-

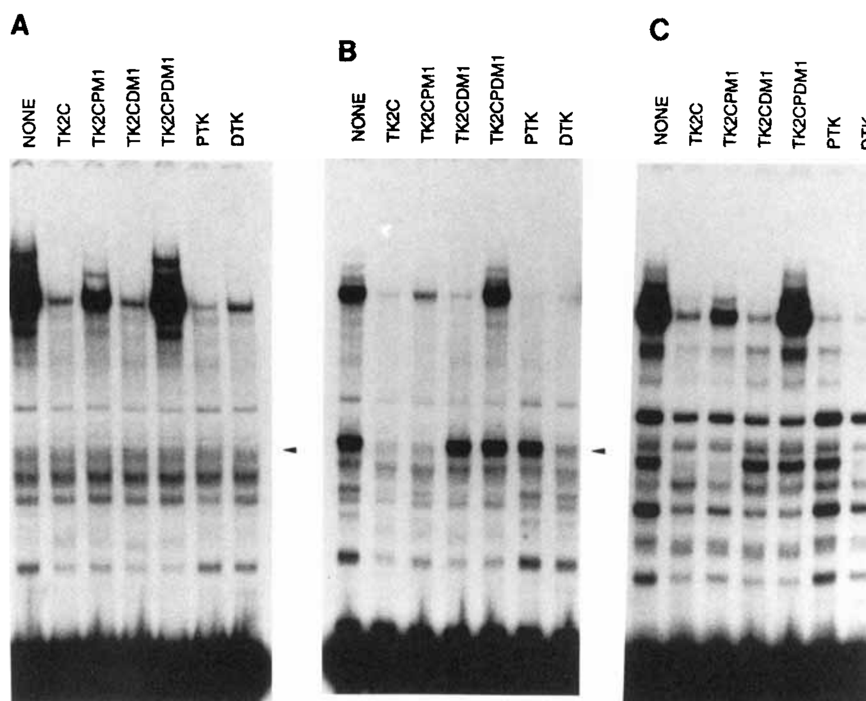

Fig. 7. Competition for formation of the DTK-CBP complex by various competitors. Mobility shift assays were performed with the TK2C probe and nuclear extracts from BALB/c 3T3 cells (**A**), WI38 cells (**B**), or HepG2 cells (**C**). The indicated competitors (50 ng) were included in the incubation mixture to examine their ability to prevent the formation of the DTK-CBP complex. The DTK-CBP complex, or its expected position, is indicated by an arrow.

mediate response genes, such as c-myc, are normally expressed, while G_1/S phase boundary genes, such as TK or histones, are not expressed [Liu et al., 1985]. This lack of expression of G_1/S phase genes occurs despite the fact that the cells have been stimulated with serum. Failure of S phase genes to be expressed appears to be transcriptional since it is mediated by the promoter. This was demonstrated by the fact that a TK cDNA expressed by its own promoter produces no mRNA at the nonpermissive temperature, while the same TK cDNA expressed by an SV40 promoter produces more mRNA than at the permissive temperature [Lipson et al., 1989]. Therefore, such ts mutant cell lines are useful for determining if an S phase-specific gene is correctly cell cycle-regulated [Kim et al., 1988; Lipson et al., 1989].

CCAAT Motifs in the Human TK Gene Promoter

Several studies have examined the CCAAT motifs in the human TK gene promoter, and the proteins that bind to them. Deletion analyses have demonstrated that they contribute to promoter strength [Kreidberg and Kelly, 1986; Lipson et al., 1989; Arcot et al., 1989]. Some studies have implicated them in regulation of the cell cycle-dependent expression of TK mRNA, since different amounts of protein binding was observed in mobility shift experiments with nuclear extracts from quiescent or serum stimulated cells [Knight et al., 1987; Pang and Chen, 1993]. Not all investigators who have examined protein binding to TK CCAAT boxes, however, have observed similar cell cycle-dependent protein binding [Arcot et al., 1989]. There has also been some controversy regarding the identity of the protein that binds to the TK CCAAT boxes: Arcot et al. [1989] have suggested that the CCAAT-binding protein has the characteristics of NF-Y based on its relative affinity for various mobility shift competitors, while Pang and Chen [1993] have suggested a unique identity for the TK CCAAT-binding protein based on its physical properties. Recently, NF-Y was identified as binding to the human TK CCAAT motifs by purification and western blotting [Chang and Liu, 1994]. Chang and Liu [1994] also demonstrated that the abundance of the A subunit of NF-Y decreased with serum deprivation of IMR-90, but not of HL-60, cells. This latter observation might explain why different results concerning cell cycle regulation are obtained in different laboratories using different cell lines.

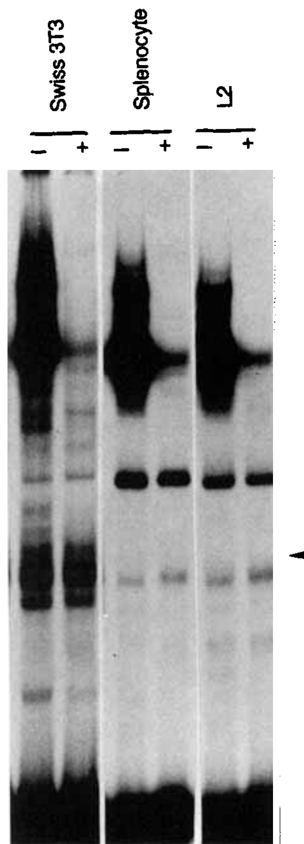

Fig. 8. Examination of other mouse cells for the presence of DTK-CBP. Mobility shift assays were performed with the TK2C probe and nuclear extracts from serum-stimulated Swiss 3T3 cells, concanavalin A-stimulated, primary mouse splenocytes, or IL-2-stimulated L2 cells. The presence or absence of 50 ng of unlabeled probe in the incubation mixture is indicated above each lane by + or −, respectively. Specific protein binding is defined by the ability of such an excess of competitor to prevent the formation of a complex. The position where the DTK-CBP complex would be expected is indicated by an arrow.

In previous experiments, we have demonstrated that both CCAAT boxes of the human TK gene promoter contribute to TK mRNA expression, but that the two motifs were not entirely equivalent or interchangeable [Mao et al., 1995]. In this work, we have examined protein binding to the two CCAAT boxes to begin to investigate why the two CCAAT boxes are not equivalent.

Protein Binding to Individual TK CCAAT Box Probes

Using probes containing the individual CCAAT motifs, we confirmed the observations of Arcot et al. [1989]. The same abundant protein(s) appeared to complex with both the proximal and distal CCAAT of the human TK gene promoter (Fig. 1), and to have a higher affinity for the proximal CCAAT (not shown). Comparison of the relative affinity of various competitors (Fig. 2) suggested that the protein that forms the abundant complexes with the TK CCAAT probes has a similar competitive profile as NF-Y [Dorn et al., 1987; Arcot et al., 1989]. We have not examined the physical properties of this protein since it is not necessary for similar proteins from different species to have identical physical characteristics. Therefore, it may not be possible to directly compare the protein we observe with CBP/tk [Pang and Chen, 1993]. Based on the work of Chang and Liu [1994], however, it is likely that this protein is NF-Y, and that all laboratories have observed the same protein since there is only one abundant complex that is formed with the TK CCAAT box probes, and which would be observed with short autoradiographic exposures.

The long autoradiographic exposures we have used suggest that the abundant CCAAT binding protein does not fluctuate dramatically with cell physiology. However, shorter exposures might make the binding of this protein appear to be more responsive to the state of cell stimulation. In addition, the abundance of the various complexes might change if equal volumes of nuclear extract (equal numbers of cells if protein recovery is equivalent) were used instead of equal amounts of nuclear extract (5 μg/lane). It is not clear which protocol would provide the most correct picture. Therefore, it is not clear what significance can be attributed to the observation of small changes in complex abundance. There is no question, however, that the abundant CCAAT binding protein is present in TK$^-$ts13 cells exposed to all three conditions from which nuclear extracts were isolated.

Protein Binding To Probes With Two CCAAT Boxes

When a mobility shift probe containing two CCAAT boxes (TK2C) was used, the pattern of protein binding was more complex. At least 8 specific complexes could be identified. Of these, the smallest two were also formed with the probe in which both CCAAT boxes were mutated (TK2CPDM1, Fig. 3). The binding of such proteins may account for the previous observation of enhanced TK mRNA expression in serum-deprived cells containing a construct in which both TK CCAAT boxes were mutated [Mao et al., 1995].

The series of complexes with the least electrophoretic mobility all demonstrated a similar pattern of competition (Fig. 4, complexes a–e). There are several possible explanations for this observation: they may represent complexes of differentially spliced NF-Y [Li et al., 1992]; they may contain additional proteins that interact with the CCAAT-binding protein [Danilition et al., 1991; Milos and Zaret, 1992]; or they may contain multiple DNA binding proteins. Identification of the proteins that form these complexes will require the use of antibodies and/or purification.

The largest of the complexes formed with TK2C (complex a) was not formed when either of the CCAAT motifs was mutated. This strongly suggests that complex a represents protein binding to both CCAAT boxes simultaneously. Footprinting studies with the segment of the TK promoter surrounding the CCAAT and TATA boxes have suggested that protein binding to the proximal CCAAT box interferes with binding to the distal [Arcot and Deininger, 1992]. In support of this observation, Arcot and Deininger [1992] demonstrated that adding additional nucleotides between the two CCAAT boxes increased expression from the promoter. This presumably occurs by allowing protein binding to both CCAAT boxes simultaneously. Although the data of Arcot and Deininger are quite convincing, the mutation data presented in the previous paper clearly indicate that the distal CCAAT contributes to TK mRNA expression, and the observation of complex a formation in this work, demonstrates that this may occur through simultaneous protein binding to both CCAAT boxes.

Observation of a Protein That Can Only Bind to the Distal CCAAT

The competitive profile of complexes a–e were similar to those observed with the single CCAAT probes. This suggests that the same protein is responsible for formation of these complexes. In contrast, two independent experiments demonstrate that a unique protein is responsible for formation of complex f: only competitors containing an intact distal CCAAT were able to compete for formation of complex f; only probes containing an intact distal CCAAT were able to form complex f. Together, these observations indicate that the protein responsible for formation of complex f, DTK-CBP, binds exclusively to the distal CCAAT of the human TK gene promoter. The reason that this protein was not previously identified is not readily apparent. However, it is likely that the shorter probes did not contain all of the bases necessary for strong protein binding, or that these bases were too close to one end of the probe for effective binding. The binding site specificity of this protein is currently being examined.

DTK-CBP in Other Cell Lines

Comparison of complexes formed in mobility shift assays with the TK2C probe and nuclear extracts from several different types of cells (Fig. 5 and 8) clearly demonstrates that multiple CCAAT binding proteins can recognize the same CCAAT sequence [Johnson and McKnight, 1989]. The pattern of complexes formed with nuclear extracts from each cell line was distinct, and in many cases, was dependent on the physiology of the cell (not shown). Although many nuclear extracts formed complexes of similar electrophoretic mobility, the abundance of each complex varied with the cell. These observations suggest that cells might regulate gene transcription by competition of proteins with overlapping binding specificities but different efficacies or actions. By controlling the expression of these various competing CCAAT binding proteins, the expression of a gene might vary between different cell types without a requirement for tissue specific transcription factors.

The observations described here suggest that DTK-CBP is not present in several different types of mouse cells, or if present, it is extremely rare. In contrast, DTK-CBP is significantly more abundant in two different human cell lines than in the hamster fibroblasts in which it was first observed. This could suggest that DTK-CBP has a significant role in modulating human TK mRNA expression in cells that express this protein. However, the fact that it does not appear to be present in BALB/c cells, in which transcriptional regulation has been demonstrated to contribute to cell cycle specific expression of murine TK mRNA [Coppock and Pardee, 1987; Friedovich-Keil et al., 1991], indicates that this protein is unlikely to be essential for cell cycle regulation. This speculation is further supported by the fact that the Chinese hamster TK gene is known to lack a distal CCAAT box [Lewis, 1986], while the mouse TK gene promoter contains no CCAAT motifs [Lieberman et al., 1988; Seiser et al., 1989]. In addition, previous data suggests that TK mRNA appears to be correctly regu-

lated in TK$^-$ts13 cells when the distal CCAAT box is inactivated [Mao et al., 1995]. Thus, this protein may play a role in contributing to the strength of the human TK promoter, but it probably does not control cell cycle specific transcriptional regulation of TK mRNA expression.

SUMMARY

The pattern of protein binding to the longer probe containing both CCAAT boxes is more likely to represent the in vivo situation than binding to the shorter probes with only one CCAAT. Thus, the proteins that form complexes b–e in mobility shift assays are likely to be preferentially associated with the proximal CCAAT. The same proteins are capable of associating with the distal CCAAT, but appear to do so with a weaker affinity. Another protein that binds exclusively to the distal CCAAT, which we have designated DTK-CBP, is likely to compete for binding to this site. In addition, the observation of complex a, which could only be formed with the TK2C probe containing both CCAAT boxes, suggests that proteins can bind to both CCAAT motifs simultaneously. Identification of these proteins will require further investigation. However, it is likely that the DTK-CBP is responsible for the previous observation that the two CCAAT motifs are not functionally equivalent, and that the distal CCAAT has an important role in contributing to promoter strength [Mao et al., 1995], especially in cells that abundantly express this protein.

ACKNOWLEDGEMENTS

This work was supported in part by grant 1 PO1 CA56309 from the National Institutes of Health.

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