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CCAAT-Box Contributions to Human Thymidine Kinase mRNA Expression

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Abstract In order to examine the role of two inverted CCAAT boxes near the start of transcription of the human thymidine kinase (TK) gene, a series of constructs were prepared in which one or both CCAAT boxes were deleted or mutated. These altered promoters (1.2 kb of 5′-flanking sequence) were used to express a TK minigene containing the first two exons and introns followed by the remainder of the cDNA. RNA blots were prepared from stable cell lines of ts13 cells containing these constructs under three conditions: 1) serum deprived cells, 2) serum stimulated cells, and 3) cells that had been stimulated with serum, but were arrested in the G_1 phase of the cell cycle by the temperature sensitive mutation carried by these cells. TK mRNA expression from each construct was suppressed by the temperature sensitive block to cell cycle progression. Measurement of protein expression from the various altered TK promoters indicated that both CCAAT boxes contribute to promoter strength. These experiments also suggested that the two CCAAT boxes were not equivalent and that the distal CCAAT could substitute for the proximal CCAAT, but the converse was not true.

Key words: transcription, site-directed mutagenesis, promoter, DNA-binding proteins, cell cycle, S phase

Thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) is a pyrimidine salvage enzyme that is expressed coordinately with other proteins and enzymes involved in cellular DNA synthesis. The amount of TK protein and activity increases sharply 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; Subblefield and Muller, 1965; Liu et al., 1985]. Steady-state levels of TK mRNA also increase sharply at the onset of S phase when serum-deprived or quiescent cells are stimulated to proliferate [Liu et al., 1985; Stuart et al., 1985; Coppock and Pardee, 1987]. Therefore, TK has been actively investigated as a model for cell cycle-specific, coordinate expression of G_1/S phase boundary genes.

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The regulation of TK expression is complex and occurs at transcriptional, posttranscriptional, translational, and posttranslational levels [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]. Which mode of regulation dominates may depend on the physiology of the cell. For example, in quiescent fibroblasts stimulated to proliferate, transcriptional activation [Coppock and Pardee, 1987; Stewart et al., 1987; Kim et al., 1988; Lipson and Baserga, 1989] must precede the appearance of TK protein and activity. In continuously cycling cells [Sherley and Kelley, 1988; Kauffman and Kelly, 1991] or in cells that cease proliferating upon induction of differentiation [Gross and Merrill, 1988, 1989; Chang, 1990; Kauffman and Kelly, 1991], however, posttranscriptional, translational, and posttranslational regulation more acutely control the

amount of TK enzyme and activity [Kauffman et al., 1991].

It has been reported that the 5′-flanking sequences of the human TK gene contribute significantly to the S-phase-specific expression of TK mRNA [Travali et al., 1988; Lipson et al., 1989; Roehl and Conrad, 1990]. The cis-acting regulatory element responsible for cell cycle transcriptional regulation has been localized to a 20 bp segment approximately 100 bp 5′ of the cap-site [Kim and Lee, 1991, 1992].

Among the many putative cis-regulatory elements within the TK gene 5′-flanking sequence [Kreidberg and Kelly, 1986; Flemington et al., 1987; Arcot et al., 1989; Sauvé et al., 1990], binding sites for SP1 and CCAAT boxes have been demonstrated to be important determinants of the amount of TK mRNA produced [Arcot et al., 1989]. The inverted CCAAT elements located near the start of TK transcription were also reported to interact with a DNA-binding protein in a cell cycle-specific manner, implicating them in S phase-specific regulation of TK mRNA expression [Knight et al., 1987; Pang and Chen, 1993]. Competition experiments suggested that NF-Y may be the CCAAT-binding protein that binds to these elements in the TK promoter [Arcot et al., 1989]. This was subsequently confirmed by western blotting, and it was demonstrated that expression of the A subunit of NF-Y is serum dependent in IMR-90, but not in HL-60 cells [Chang and Liu, 1994].

Several eucaryotic putative CCAAT-binding proteins have already been cloned, including NFI (CTF) [Santoro et al., 1988], C/EBP [Landschulz et al., 1988], NF-Y (CP1, CBF) [van Huijsduijnen et al., 1990], YB-1 [Didier et al., 1988; Sakura et al., 1988; Tafuri and Wolfe, 1990], NFI-B [Inoue et al., 1990], and LAP [Descombes et al., 1990]. They represent a diverse group of proteins, some of which require heterologous subunits for activity [Chodosh et al., 1988a,b]. Many other CCAAT-binding proteins have been identified or purified from various tissues [for reviews see Dynan and Tjian, 1985; Maniatis et al., 1987; Johnson and McKnight, 1989; Mitchell and Tjian, 1989], but their relationship to the cloned factors has not yet been determined.

Some CCAAT-binding proteins are enriched in specific tissues and probably contribute to tissue specific gene expression [Inoue et al., 1990; Descombes et al., 1990; Maniatis et al., 1987; Hwang et al., 1990]. Other CCAAT-binding proteins have been implicated in cell cycle regulation of histone gene expression [Gallinari et al., 1989; La Bella et al., 1989]. However, transcriptional regulation by CCAAT-binding proteins is complicated by the fact that multiple CCAAT-binding proteins can coexist within the same cell [Chodosh et al., 1988a; Johnson and McKnight, 1989; Dorn et al., 1987; Raymondjean et al., 1988] and that their binding specificities overlap [Johnson and McKnight, 1989]. The mechanisms by which these coexisting, overlapping activities properly regulate gene expression has not yet been determined.

In this paper, we have examined the contribution of CCAAT-binding proteins to TK mRNA expression. Our results suggest that the two inverted CCAAT elements located near the start of TK transcription are important for TK mRNA expression, but they are not equivalent.

MATERIALS AND METHODS

Plasmid Construction

The plasmids shown in Figure 1 were constructed from p-1.2B5′TKTKi [Lipson et al., 1989]. This parental plasmid contains a TK minigene composed of the first two exons and introns of the human TK gene, completed by the remainder of the cDNA, and expressed by approximately 1.2 kbp of human TK 5′-flanking sequences [Sauvé et al., 1990]. The distal CCAAT box was deleted between the unique restriction endonuclease sites for Eag I and Nae I to form p-1.2 (-D) TKi. The proximal CCAAT box was deleted by ligating a PCR amplification product that initiated several bases 5′ of the noncanonical TATA box, between the Nae I restriction endonuclease site and the Apa I site in the second intron to form p-1.2 (-P) TKi. Deletion of both CCAAT boxes resulted from ligation of the same PCR amplification product between Eag I and Apa I to form p-1.2 (-P-D) TKi.

The proximal and distal CCAAT boxes were inactivated by site-directed mutagenesis, using PCR primers [Higuchi et al., 1988] that converted the inverted CCAAT to CTGAT. PCR products produced with these mutant primers were ligated between the Apa I site in the second intron and either the Nae I site (p-1.2 (PM1) TKi) or the Eag I site (p-1.2 (DM1) TKi) to mutate the proximal or distal CCAAT boxes, respectively. The plasmid with two mutant CCAAT boxes, p-1.2 (PDM1) TKi, was constructed from p-1.2 (PM1) TKi. The correct

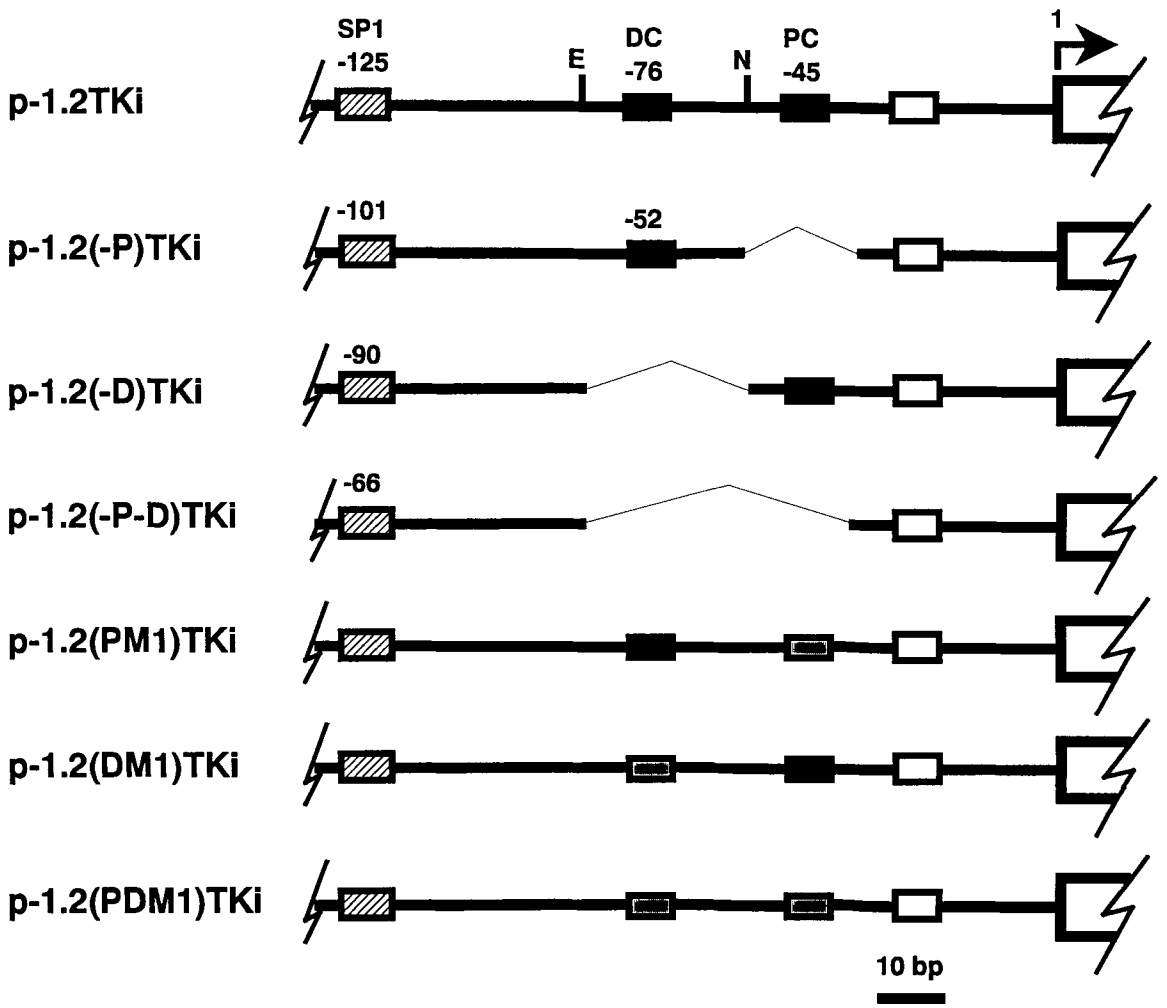

Fig. 1. TK promoter deletions and mutations. The names of the plasmids are shown to the left of the schematics of the promoter structures. The line represents 5'-flanking sequence and the large open bar that is cut at the right represents the beginning of exon 1. The transcription initiation site is indicated by the rightward arrow and is numbered 1. The small open box, the filled boxes, and the crosshatched boxes represent the noncanonical TATA, inverted CCAAT, and GGGCGG boxes, respectively. Stippled boxes represent inverted CCAAT boxes that have been mutated to CTGAT. The positions of the proximal CCAAT (PC), distal CCAAT (DC), and GGGCGG (SP1 for SP1 binding site) are indicated above the parental plasmid and in the other plasmids only if the position is altered by element excision. The unique EagI and NaeI restriction enzyme sites are also shown above the parental plasmid. The parental plasmid, p-1.2TKi, consisted of 1.2 Kb of human TK 5'-flanking sequence expressing a minigene composed of the first two exons and introns of the human TK gene and completed by the remainder of the cDNA (identical to p-1.2B5'TKTKi in Lipson et al., 1989). Deletion of the proximal or distal CCAAT box produced the plasmids p-1.2 (-P) TKi or p-1.2 (-D) TKi, respectively. Deletion of both CCAAT boxes produced p-1.2 (-P-D) TKi. Mutation of the proximal or distal CCAAT boxes produced p-1.2 (PM1) TKi or p-1.2 (DM1) TKi, respectively. Mutation of both CCAAT elements produced p-1.2 (PDM1) TKi.

identity of each plasmid was confirmed by dideoxy-termination sequencing [Sanger et al., 1977].

A second series of constructs were prepared to measure promoter strength. Approximately 460 bases of the TK promoter containing the various mutations/deletions of the two CCAAT elements were amplified by PCR and then subcloned into the Hind III site of pGL2-basic (Promega). The sequence of the TK promoter in each construct was confirmed prior to transfection.

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 transfected using the cationic liposome forming reagent, lipofectin (BRL) [Felgner et al., 1987].

Stable TK transformants were selected at 34°C in gHAT medium (glycine, hypoxanthine, aminopterin, thymidine medium) supplemented with 10% calf serum. The established cell lines were a mixture of several hundred clones and were always maintained in gHAT medium at the permissive temperature of 34°C. The cells were made quiescent by a combination of contact inhibition and serum deprivation (0.1%) for 48 h prior to stimulation.

RNA Extraction and RNA Blots

Total RNA was extracted from the cells with guanidine thiocyanate [Chomczynski and Sacchi, 1987]. RNA blots were prepared after fractionation of 20 μg of total RNA on agarose gels containing formaldehyde [Thomas, 1980; Sambrook et al., 1989] and hybridized with radioactive probes prepared by random priming [Feinberg and Vogelstein, 1983].

Luciferase Assays

Promoter strengths were determined by transient expression of luciferase [de Wet et al., 1987]. TK⁻ts13 cells were cotransfected with 2 μg of a TK-luciferase construct and 2 μg of pSV-β-galactosidase control plasmid (Promega), as described above. Cytoplasmic extracts were isolated from the transfected cells after 48 h, by 3 freeze/thaw cycles in a hypotonic medium (50 mM potassium phosphate, pH 7.8, 1 mM DTT, 0.5 mM EDTA, 1 μg/ml leupeptin). Luciferase activities were measured for 60 s in a Lab-Systems Luminoskan microtiter plate luminometer using 20 μl of cytoplasmic extract and 80 μl of luciferase assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP). Luciferase activities were normalized to β-galactosidase activities in order to minimize variability due to efficiencies of transfection or extract isolation. β-galactosidase was assayed by incubating 20 μl of cytoplasmic extract in a total volume of 100 μl of β-gal assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 0.67 mg/ml PNPG [para-nitrophenyl-galactoside, GIBCO/BRL]) at 37°C until some yellow color could be observed. The reactions were stopped by the addition of 150 μl of 1 M Na_2CO_3, and the absorbance at 405 nm was measured with an SLT CR400 microtiter plate reader. Transfections were performed on 3 different days and both luciferase and β-galactosidase activities were measured in duplicate from each transfection.

RESULTS

TK constructs with various deletions or mutations of the two inverted CCAAT boxes (Fig. 1) were prepared and transfected into the TK deficient variant of the Syrian hamster cell line ts13. This cell line has a temperature sensitive mutation in cell cycle progression such that the cells arrest in the G_1 phase of the cell cycle when grown at the nonpermissive temperature of 39.6°C [Liu et al., 1985]. Cell cycle specific temperature sensitive mutants, such as ts13, have been used to help define proper phase specific gene expression [Kim et al., 1988].

Cells containing the transfected constructs were selected by reversion of the TK deficient phenotype and a mixed population of stable cell lines was used for RNA blotting (Fig. 2). RNA was isolated from each cell line under three conditions: 1) confluent cells that were serum-deprived for 72 h; 2) cells that were serum-deprived for 48 h and then stimulated with medium containing 10% serum for 24 h at the permissive temperature of 34°C; and 3) cells treated as in 2), but serum-stimulated at the nonpermissive temperature of 39.6°C for 24 h. RNA blots were hybridized with radioactive probes for TK and histone H3 as a marker for S phase. The cell line containing the parental plasmid (Fig. 2A) exhibited the expected regulation of TK mRNA: low expression in serum-deprived cells (lane 1) that increased with stimulation (lane 2) but was suppressed by the temperature sensitive block to cell cycle progression (lane 3). Cells containing constructs with a deletion of one or both CCAAT boxes or a mutation of either CCAAT box had similar regulation of steady-state TK mRNA expression (Fig. 2B–F). Cells containing the construct with mutations of both CCAAT boxes exhibited higher levels of TK mRNA in serum-deprived cells than cells with the other constructs (Fig. 2G). However, TK mRNA levels were suppressed in this cell line when stimulated with serum at the nonpermissive temperature (Fig. 2G, lane 3).

The observation of high levels of TK mRNA in quiescent cells containing the construct with two CCAAT mutations was unexpected. This observation was confirmed by preparation of another mixed population of cell lines containing the double CCAAT mutation by independent

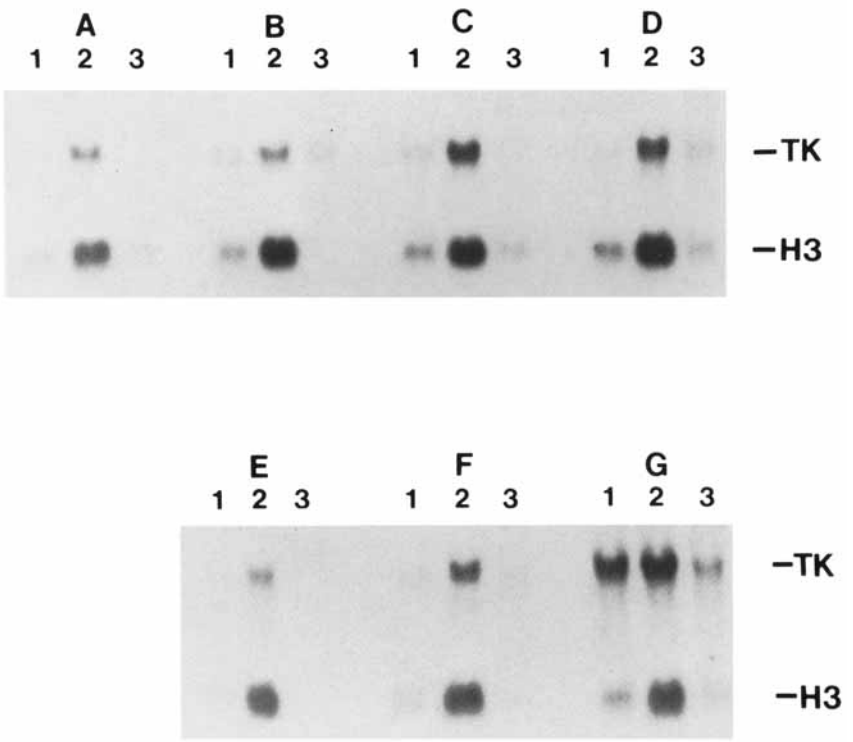

Fig. 2. Regulation of TK mRNA expression. RNA was isolated from pools of stable lines of TK⁻ts13 cells containing the constructs indicated in Figure 1. For each cell line, lane 1 contains RNA from serum deprived cells, lane 2 contains RNA from cells that were stimulated with serum at the permissive temperature for 24 h, and lane 3 contains RNA from cells that were stimulated with serum for 24 h at the nonpermissive temperature of 39.6°C. The cell lines represented in each panel and the average number of copies of each construct per cell (as determined by Southern (1975) blotting) are **A:** p-1.2TKi, 15 copies/cell; **B:** p-1.2 (-P) TKi, 45 copies/cell; **C:** p-1.2 (-D) TKi, 35 copies/cell; **D:** p-1.2 (-P-D) TKi, 80 copies/cell; **E:** p-1.2 (PM1) TKi, 50 copies/cell; **F:** p-1.2 (DM1) TKi, 40 copies/cell; **G,** p-1.2 (PDM1) TKi, 90 copies/cell.

transfection and selection of the same construct or a newly prepared construct (not shown).

To determine the relative importance of each CCAAT box to TK promoter strength, constructs were prepared in which approximately 460 bases of TK 5′-flanking sequences containing the deletions or mutations shown in Figure 1 were used to transiently express a luciferase cDNA. These values are shown in the histogram in Figure 3, where 100% was defined as the strength of the parental construct (p-0.5TKi). Deletion of the proximal CCAAT box (p-0.5 (-P) TKi) had no significant effect on luciferase expression. In contrast, deletion of the distal CCAAT box (p-0.5 (-D) TKi) reduced luciferase expression to about 55% of that by the parental promoter. Deletion of both CCAAT boxes (p-0.5 (-P-D) TKi) further reduced expression from the TK promoter to approximately 30% of its normal strength.

Mutation of either the proximal (p-0.5 (PM1) TKi) or distal (p-0.5 (DM1) TKi) CCAAT reduced TK promoter strength to about 45% or 40%, respectively, of that of the native promoter. Expression from the TK promoter with two CCAAT box mutations (p-0.5 (PDM1) TKi) was also about 40% of that from the unmutated promoter.

DISCUSSION

Gene Promoters

Many promoters for genes that are transcribed by RNA polymerase II contain three motifs for sequence-specific DNA binding proteins: TATAA, GGGCGG, and/or CCAAT [Breathnach and Chambon, 1981]. The TATAA box, if present, is usually located approximately 30 bp 5′ of the start of transcription. Two CCAAT or GGGCGG boxes are usually found between 40 and 110 bp 5′ of the transcription initiation site [Breathnach and Chambon, 1981]. In addition, a wide variety of other elements that can contribute to the regulation of transcription have been identified. These elements can be located farther upstream, downstream, or within the

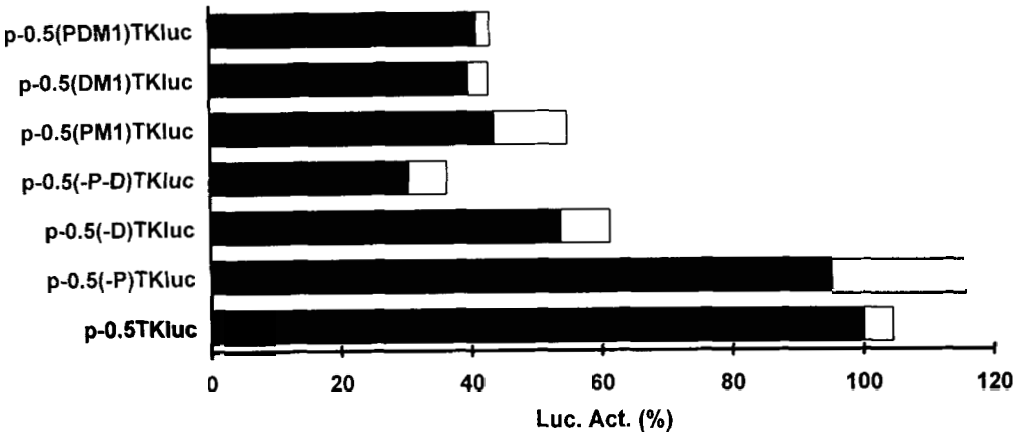

Fig. 3. TK promoter strength. Constructs were prepared in which 460 bp long TK promoters containing the various CCAAT deletions or mutations were used to express a luciferase cDNA. These plasmids were then cotransfected with a plasmid expressing β-galactosidase from an SV40 promoter into TK⁻ ts13 cells. Luciferase activity was assayed 48 h after transfection and normalized to the activity of β-galactosidase in order to minimize variability due to transfection efficiency. The solid bars in the histogram indicate the average (n = 3), relative amount of luciferase activity (protein) produced by each plasmid where the amount of luciferase activity in the parental plasmid was assigned as 100%; the open bars indicate the standard error.

gene being regulated, and can stimulate or inhibit transcription [Maniatis et al., 1987; Johnson and McKnight, 1989; Mitchell and Tjian, 1989].

Expression of the Human TK Gene

The human TK gene fits into this paradigm. TK transcription initiates 28 bp 3′ of the first T of the noncanonical TATA box (TTTAAA) (X. Mao, unpublished results). The TK gene 5′ flanking sequence also contains two inverted CCAAT boxes located at −45 and −76 relative to this transcription initiation site, numerous GGGCGG elements at various distances and putative binding sites for a variety of other DNA-binding proteins that regulate transcription [Kreidberg and Kelly, 1986; Flemington et al., 1987; Arcot et al., 1989; Sauvé et al., 1990]. Deletion analyses have demonstrated that the CCAAT and GGGCGG elements contribute to the strength of the TK promoter [Arcot et al., 1989].

Although TK expression is complex and regulated by several posttranscriptional mechanisms [Gudas et al., 1988; Sherley and Kelly, 1988; Gross and Merrill, 1988, 1989; Chang, 1990; Ito and Conrad, 1990; Kauffman and Kelly, 1991; Kauffman et al., 1991], it has been shown that transcriptional regulation also contributes to the S phase-specific expression of TK mRNA, particularly in quiescent cells stimulated to proliferate [Coppock and Pardee, 1987; Stewart et al., 1987; Kim et al., 1988; Travali et al., 1988; Lipson and Baserga, 1989]. A location for the cis-element that mediates cell cycle regulated transcription has been reported [Kim and Lee, 1991, 1992], and some of the proteins that bind to this element have been identified [Li et al., 1993]. A protein in nuclear extracts from BALB/c cells has been observed to bind to the CCAAT boxes of the human TK gene in a cell-cycle-specific manner, suggesting that proteins that bind to the CCAAT boxes might regulate S-phase-specific transcription of TK [Knight et al., 1987]. Similarly, a protein in nuclear extracts of IMR-90 cells was observed to bind in a cell-cycle-specific manner [Pang and Chen, 1993], which was demonstrated to be NF-Y [Chang and Liu, 1994]. However, a similar cell-cycle-specific CCAAT-binding activity was not observed in nuclear extracts of HeLa cells [Arcot et al., 1989].

The Model

To assess the role of the two inverted CCAAT boxes in the regulation of TK mRNA expression, we excised or mutated one or both CCAAT motifs within a promoter that included 1.2 Kb of 5′ flanking sequence [Sauvé et al., 1990]. These altered promoters were used to express a TK minigene in TK⁻ts13 cells, which contain a temperature sensitive mutation in cell cycle progression so that they arrest in the second half of G_1 at the nonpermissive temperature. When these cells are arrested at the nonpermissive temperature, immediate response genes such as c-myc are normally expressed, while G_1/S phase boundary genes, such as TK or histones, are not ex-

pressed [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. Furthermore, the failure of S phase genes to be expressed appears to be transcriptional since it is mediated by the promoter. Thus, 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].

The Effect of CCAAT Mutation

The data indicate that TK mRNA expression was enhanced by serum stimulation in all but one of the cell lines and was lower in each of the various cell lines stimulated at the nonpermissive temperature, than at the permissive temperature. The exception to this general observation was in the cell line containing the construct in which both CCAAT boxes were mutated. TK mRNA expression in this cell line was very high in the serum-deprived condition and very little stimulation was observed at the permissive temperature. As mentioned above, however, TK mRNA expression was reduced when the cells containing this construct were stimulated with serum at the nonpermissive temperature. This observation was confirmed in two independent transfection and selection processes using the same construct or an independently prepared construct, indicating that the observed behavior is characteristic of this construct.

Possible Explanations for High mRNA Expression in Serum-Deprived Cells Containing the Double CCAAT Mutation

There are at least two possible explanations for the observation of higher levels of TK mRNA expression in serum deprived cells containing the construct with both CCAAT boxes mutated: 1) TK CCAAT boxes mediate inhibition of TK mRNA expression in serum deprived cells; 2) other proteins may be able to bind to sequences at or near the mutated CCAAT boxes to stimulate TK mRNA expression in serum deprived cells.

It is possible that the TK CCAAT boxes regulate suppression of TK mRNA transcription in serum deprived cells. There have been some reports of CCAAT elements inhibiting mRNA expression [Alder et al., 1992; Barberis et al., 1987]. However, most CCAAT boxes have been demonstrated to stimulate transcription. Furthermore, the decrease of expression from the TK promoter when the CCAAT boxes are mutated suggest that proteins which bind to these CCAAT motifs stimulate TK transcription. Nevertheless, an activating protein could suppress transcription if it bound constitutively and its activating function required induction by ligand binding or posttranslational modifications such as phosphorylation or dephosphorylation.

It is also possible that the loss of suppression in the construct with two mutated CCAAT boxes may result from the inability of CCAAT-binding proteins to interact with the promoter, thereby allowing another protein to bind and activate transcription. A similar displacement mechanism has been reported to regulate CCAAT-binding protein interaction with several gene promoters [Barberis et al., 1987; Skalnik et al., 1991]. In the situation described here, the CCAAT-binding protein would be a negative regulator and act as a displacement protein for an activator.

A slight variation on this theme is the possibility that mutation of the CCAAT box created a new protein binding site. There is little evidence to either support or refute this alternative at the present time. The subsequent paper will show that some proteins can bind to a mobility shift probe containing two mutated CCAAT motifs (as well as two normal CCAAT motifs), but the identity or role of these proteins remains unknown.

The Effect of CCAAT Deletion

The observation of high levels of TK mRNA in serum-deprived cells containing the construct with both CCAAT boxes mutated was different from the cell line containing the construct in which both CCAAT boxes were excised. If the latter speculation is correct, it is likely that the binding site for the putative protein(s) responsible for high levels of TK mRNA expression in serum-deprived cells was deleted during excision of the CCAAT boxes, or was not created since these sequences were absent.

Contribution of CCAAT Boxes to Cell Cycle Regulated TK mRNA Expression

In the absence of other complications, the observation that TK mRNA expression de-

creased when TK⁻ts13 cells containing the various constructs were stimulated with serum at the nonpermissive temperature would be interpreted as indicating that neither CCAAT box is the sole determinant of cell cycle-regulated TK mRNA expression. However, the anomaly observed in cells containing the construct with two CCAAT mutations suggests that different mechanisms may account for suppression of G_1/S phase genes in G_0 or early G_1 induced by serum deprivation, and in a temperature sensitive, mid-G_1 block to cell cycle progression. This speculation is consistent with recent evidence that cessation of proliferation induced by different physiological manipulations is accompanied by differences in gene expression [Gustincich and Schneider, 1993].

Contribution of CCAAT Boxes to TK Promoter Strength

Quantitation of gene expression from TK promoters containing mutated CCAAT motifs conclusively demonstrates that both contribute to the strength of the TK promoter. Mutation of either or both CCAAT boxes yielded a promoter that was only about 40% as strong as the same promoter with intact CCAAT boxes.

The effects of CCAAT excision were not identical to those of mutation. Excision of the proximal CCAAT did not significantly alter expression from the TK promoter, while mutation of this motif caused a marked reduction of expression. In contrast, expression was reduced when the distal CCAAT was excised, but not as much as when it was mutated. The quantitative differences between excision and mutation probably arise from the fact that when the CCAAT boxes are removed, other protein binding sites can partially or completely substitute for the missing segment [Tronche et al., 1991]. When the proximal CCAAT is excised, the distal CCAAT is located in a location that is close to that of the proximal CCAAT with respect to the site where transcription starts. The SP1 binding site, which substitutes for CCAAT elements in some promoters [Breathnach and Chambon, 1981] also moves closer to the capsite. However, deletion of both CCAAT boxes severely impairs promoter strength, despite the fact that the SP1 binding site is located approximately 66 bp 5′ of the capsite when both CCAAT elements are excised. Together, these observations suggest that the distal CCAAT can substitute for the proximal CCAAT, but the reverse is not true. In addition, promoter strength is determined to a large part by the presence of one of the CCAAT boxes 40–50 bp 5′ of the start of transcription. Furthermore, if the SP1 binding site is located closer to the capsite, it can act as a partial surogate for a missing second CCAAT. However, only the presence of an SP1 binding site about 65 bp 5′ of the capsite is not a good substitute for a CCAAT box 40–50 bp from the capsite.

SUMMARY

In summary, we have demonstrated that both of the CCAAT boxes of the human TK promoter are important for the efficient expression of TK mRNA. The two inverted CCAAT elements do not have identical activities and excision of the distal CCAAT is more deleterious than that of the proximal CCAAT.

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