Cell cycle regulatory element in the promoter of the human thymidine kinase gene and its binding to factors

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When quiescent cells are stimulated to enter the cell cycle, the thymidine kinase (TK) gene is transcriptionally activated at the border of G1 and S. In this report we show that the human TK promoter contains multiple protein-binding sites. By site-directed mutagenesis, we identified a protein-binding site on the human TK promoter requited for conferring G1-S-regulated transcription to a heterologous promoter and dissociated it functionally from an adjacent protein-binding domain containing an inverted CCAAT motif requited for high basal level expression. Substitution-mutation of this site results in constitutive expression of the neo reporter gene in serum-stimulated fibroblasts, as well as in cells arrested in mid-G1 by a temperature-sensitive mutation. The regulatory domains for the human TK promoter exhibit interesting symmetrical features, including a set of CCAAT motifs and sites similar to the novel Yi protein-binding site recently discovered in the mouse TK promoter. Thus, components of the hTK complex is important for hTK gene regulation.

INTRODUCTION

A fundamental problem in cell biology has been the study of how cell cycle is controlled. In mammalian cells, cellular proliferation involves multiple control steps (1,2,3). When quiescent cells are stimulated to enter the cell cycle in response to physiological stimuli such as growth factors,. the initial regulatory events direct the transition of cells from quiescence into the G1 phase. Several immediate early growth responsive genes and their products have been implicated as the primary mediators of this signal transduction pathway (4). The next regulatory step directs the stimulated, competent cells through the final stages of G1 and across the G1-S boundary, and commits the cells into a new round of DNA synthesis and cell division. With the recent discovery of several mammalian cyclins active in different phases of the cell cycle (5), it has been hypothesized that cellular transcription factors, in association with the cell-cycle regulated cyclins, and their activating kinases, undergo cycles of activation and inactivation to regulate the expression of cellular proliferation-related genes (6).

Previously, we have demonstrated that the human TK promoter sequence can confer G1-S control on a heterologous, non-cell-cycle regulated promoter in an orientation dependent manner (7). Further 5′ deletion reveals that a 70 base pair(bp) fragment, spanning between -133 to -64, is sufficient for the S-phase dependent control (11). However, the critical information of the functional domain(s) required for G1/S control in vivo is lacking. In this report, we defined the protein binding domains of the proximal human TK promoter, using DNase I protection analysis. We identify here a protein binding site required for the G1-S regulated transcription of the human TK promoter in vivo. We were able to dissociate the G1-S control domain from an adjacent protein binding protein required for enhancing activity(9,11). Mutation of this regulatory site eliminates the ability to confer G1-S regulated transcription onto a heterologous promoter in serum stimulated cells. It also results in constitutive expression of the neo reporter gene in cells arrested in mid G1 by a temperature sensitive mutation. Special features of the human TK regulatory domains are discussed.

MATERLALS AND METHODS

Cell, Culture and Gene Transfection - Conditions for culturing, synchronization and transfection of the Chinese hamster fibroblast cell line, K12, and measurement of DNA synthesis have been described (7, 14).

Plasmids - The construction of pTKN70M is as follows: A 47 nt synthetic oligomer spanning-133 to-87 of the coding strand with a mutated sequence at-109 to -87, was reannealed with a 43 nt synthetic oligomer spanning -106 to-64 of the non-coding strand with a mutated sequence to -106 to -84. The resultant 70 bp fragment was cloned into a 4.4 kilobase BamHI/Bg1II fragment containing the HSV *tk-neo* expression vector as previously described (7). The orientation of the fusion gene, as well as the mutated sequence (shown in Fig. 2A)were confirmed by DNA sequencing.

RNA Blot Hybridization - Conditions for RNA gel electrophoresis and blot hybridization have been described (7).

DNase I Footprint Assay - To prepare the DNA probe, pUCrd, which contains the 276 bp DraI (-241)/RsaI (+34) fragment of the human TK promoter subcloned into the SmaI site of pUC8 (with the RsaI site proximal to the EcoRI site of pUC8), was digested with EcoRI and PstI. The 294 bp EcoRI/PstI fragment was gel purified. The coding strand was 3′-end labeled at the EcoRI site by the Klenow fragment of DNA polymerase I with [a-^{32}P]dATP. Preparation of whole cell extracts was as described (15).

RESULTS

Prior to designing targets for site-directed mutagenesis to eliminate the G1-S control element, we first identified the protein-binding domains on the human TK promoter. In addition, we determined whether the binding pattern changes as quiescent cells are stimulated by serum to enter the cell cycle. For this purpose, we prepared whole cell protein extracts from synchronized K12 cells at 4,6 and 10 hr after serum release. These time points were chosen because the rate of TK gene transcription has been shown to be maximal during the border of G1 of S (13), which is between 5 and 8 hrs in K12 cells (7,11). The DNA fragment used for the analysis contained the sequence from -241 to +34 of the human TK promoter. This would allow us to detect DNA-protein interactions that are important for cell-cycle regulation within the 70 bp CCRU as well as in the surrounding sequence, including the TATA and the proximal CCAAT elements. DNase I protection assays on the coding strand revealed several DNA-binding domains (A through E) within the TK promoter (Fig. 5). For the non-coding strand, similar regions were protected although the boundaries were not identical (data not shown).

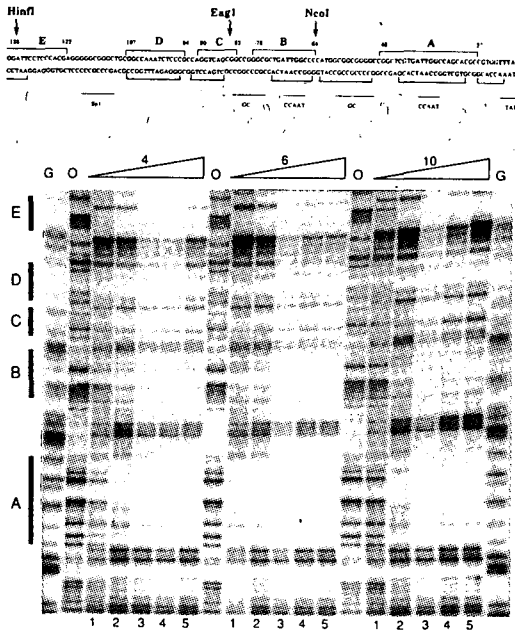

FIG. 1. **Footprint analysis of the human TK promoter.** The sequence of the human TK promoter (27) with the locations of the *HinfI*, *EagI*, and *NcoI* restriction sites is shown. Sequence motifs (TATA, CCAAT, Sp1, and GC-rich regions) are *underlined*. The DNase I-protected regions (*A–E*) are *bracketed*. The autoradiograms of the DNase I protection analysis of the coding strand are shown. The DNA probe labeled at the 3′ terminus was mixed with increasing amounts of whole cell protein extracts from synchronized K12 cells prepared at 4, 6, or 10 h after serum stimulation. *Lanes 1–5* contained 5, 10, 20, 40, and 80 μg of protein extract, respectively. No protein extract was added in *lanes O*. *Lanes G* showed the G sequencing ladder of the coding strand. The protected regions (*A–E*) are indicated.

Based on the DNase I protection analysis, within the CCRU that spans -133 to -64, there are four protein binding domains (B through E). While domain B is important for high basal-level expression, the function of domains C, D and E is unknown. A new 70 bp sequence spanning-133 to -64 was designed with a mutated sequence from -109 to -84 (Fig.2A). This mutant sequence eliminates the adjacent C and D binding domains, while maintaining the E domain and the B domain required for high basal level. This sequence was cloned into the HSV *tk-neo* plasmid to create the fusion gene, pTKN70M. Thus, this plasmid is identical in structure to the wild type fusion gene, pTKN70R, with the exception of the mutated bases spanning -109 to -84 (Fig. 2B). In contrast to pTKN50R, it contains the B and E binding domains and the mutated sequence. In previous studies, we have established that in stable pTKN70R transfectants it is capable of conferring proper G1-S control to the HSV *tk* minimal promoter (Fig. 3A,11), which by itself is non-cell-cycle regulated (7).

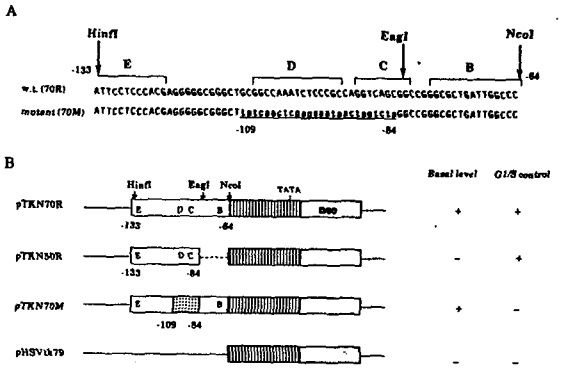

FIG. 2. **Structure of TK-neo fusion gene.** *A*, sequence of the wild type (*w.t.*) human TK promoter spanning the 70 bp between −133 and −64 is compared with that of the mutated sequence (presented in *lower case* and *underlined*). *B*, schematic drawings of the TK neo fusion genes. *Open bar*, human TK 5′-flanking sequence; *shaded bar*, neo gene coding sequence; *vertical bars*, HSV tk promoter sequence; *lines*, procaryotic vector sequence. *The 5′- and 3′-end* points of the TK sequence, the mutated sequence (□), and the location of footprinted regions *B–E* are indicated.

To determine whether domains C and D are required for this control, pTKN70M was transfected to the hamster fibroblast cell line, K12. Stable transfectants were selected either on the basis of HAT or G418. Selection by HAT eliminated any bias towards transfectants expressing high levels of *neo* transcripts. Pooled or individual transfectants were mass expanded, and synchronized by serum starvation. Upon

addition kof fresh seum, the cells progressed through the cell cycle, as demonstrated by the increase of DNA synthesis as cells entered S-phase around 12 hrs (Fig. 3B). Concomitantly, the transcript levels of the *neo* reporter gene, the endogeneous hamster histone H3.2 gene and an invariable control gene, p3A10, were measured.

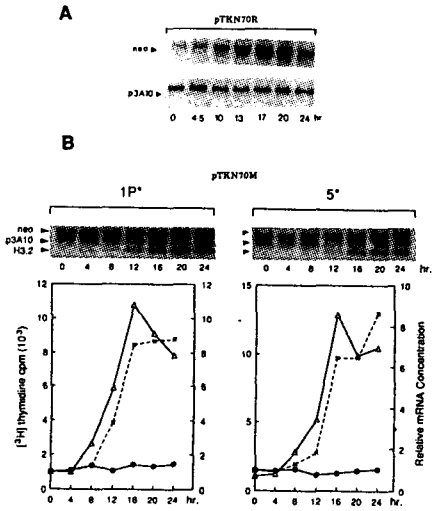

FIG. 3. **Cell cycle analysis of neo, p3A10, and histone H3.2 mRNA levels in pTKN70 stable transfectants after serum stimulation of synchronized cells.** In *A* the RNA profile of a pTKN70R stable transfectant previously described (11) is shown. In *B* RNA profiles of pTKN70M stable transfectants are shown. Sample *1P** represents pool transfectants selected by HAT, and sample *5** represents a clonal line selected by G418. The autoradiograms were quantitated by densitometry to obtain the relative levels of neo (●) and H3.2 (×), which were plotted against the rate of DNA synthesis as measured by incorporation of [^{3}H]thymidine (Δ).

The results, as shown in Fig.3B, demonstrated that the cells were well synchronized. As expected, the replication-dependent histone H3.2 transcript levels increased as cells entered into the DNA synthetic phase. In contrast, the level of the *neo* transcript was relatively constant during the cell cycle, resembling that of the invariant hamster transcript encoded by p3A10 used in our previous studies for standardization of RNA amounts in the RNA blots (7,11). The same results were obtained for transfectants selected either by HAT or G418, for pooled or individual transfectants. Therefore, within the context of the 70 bp CCRU, elimination of the C and D binding domains results in the loss of G1-S control, while maintaining a high basal level throughout the cell cycle. The results directly imply that the 25 bp region spanning -109 to -84 is required for conferring G1-S regulated transcription onto a heterologous, non-cell-cycle regulated promoter.

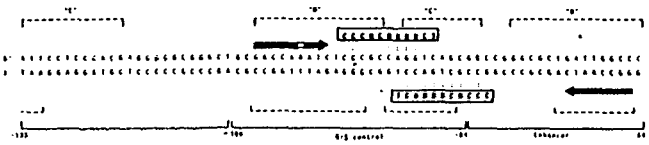

FIG. 5. **Regulatory domains of the human TK promoter.** The sequence spanning −133 to −64 is shown. The footprinted regions as described in Fig. 1 are *bracketed*. The inverted pair of repeats bearing CCAAT-like motifs is shown (*solid arrows*). The identical bases between the human TK sequence and the mouse Yi protein-binding site (*shaded box*) are connected by *dots*. The enhancer domain spans −83 to −64, the G/S control domain spans −109 to −84, and a third domain spans −133 to −110.

DISCUSSION

In this report, we provide evidence that within this 70 bp functional unit, there are multiple protein binding sites and functional domains (Fig.1). A combination of dolotion analysis and sito-mutagenesis studies directed towards these protected domains reveal the critical information that a domain spanning -109 to -84 is required for G1-S regulated transcription. Using the *neo* stable transfectants, we established that mutation of domains C and D largely eliminated the differential transcription of the TK promoter between G1 and S. This could be due to the loss of a binding site for an activator of S-phase dependent transcription. Alternatively, it could be due to the loss of a repressor site responsible for suppression of the TK promoter activity during G1 and G2. In the latter case, a mutation in domains C and D would result in a gain of promoter activity during G1 and G2. In stable transfectants, this issue cannot be accurately addressed since the copy number and the site of integration of the reporter genes have major influences on the basal promoter activities. Previously, it was shown that in contrast to longer promoter constructs, 5′ deletion to -84, when fused to a tk minigene, was unresponsive to cycloheximide (9), implying that the stringent G1/S control of the tk mRNA level was impaired by deletion of the upstream promoter sequence. These combined results are consistent with the hypothesis that domains C and D elther positively or negatively modulate the activity of the other domains such as B and E. Since the construct pTKN50R devoid of the enhancer domain B when integrated into an active chromosomal site exhibited proper G1/S regulation (11), we speculate that domains C and D can act in concert with other cellular enhancers. While the functional contribution of each individual domain remains to be determined, the successful dissociation of the S-phase regulatory domain from the adjacent enhancing comain containing the CCAAT motif provides the information necessary for the isolation of the regulatory factors interacting with these domains and the understanding of their mechanism of action.

We note, however, from our DNase I footprint analysis and previous DNA-protein gel mobility shift analysis, the hamster binding activities to the 70 bp regulatory domain remain relatively constant following serum stimulation (11). While these results apparently differ from the observed Yi binding activity in murine BALB/c3T3 A31 cells(22), the Yi binding activity is also constitutive in transformed BPA31 cells(23). In addition, in other cell-cycle regulated genes such as the histones, several regulatory factors have been identified that bind

to their target sites constitutively (3,25). Fof these factors, post-translational modifications or additional protein-protein interaction is postulated as possible mechanisms for activating the factors during the G1/S border (26). Since the TK gene is under S-phase transcriptional control in both transformed and non-transformed murine and hamster cells, the putative differences in the factor binding activities provide a unique opportunity to unravel the different molecular mechanisms that different species and cell types exercised to regulate the S-phase transcriptional control of TK gene expression.

REFERENCES

1. Baserga, R. (1985) *The Biology of Cell Reproduction* (Harvard University, Cambridge, MA),p. 134
2. Pardee,A.B. (1989)*Science* 246, 603-608
3. Naeve, G.S.,Sharma,A.,and Lee,A.S. (1991) *Current Opinion in Cell Biology* 3,261-268
4. Studzinski, G.P. (1989) *Cell Tissue Kinet.*22, 405-424
5. Pines,J. (1991) *Cell Growth and Diff*2, 305-310
6. Wagner,S.,and Green,M. (1991) *Nature* 352, 189-190
7. Kim,Y.K., Wells,S.,Lau, Y-F.C.,and Lee,A.S. (1988) Proc. Natl. Acad. Sci. U.S.A.85,5894-5898
8. Lipson,K.E.,Chen,S-T.,Koniecki,J.,Ku,D-H.,andBaserga,R.(1989) Proc.Natl.Acad.Sci.U.S.A.86, 6848-6852
9. Kim,Y.K.,and Lee,A.S. (1991) *Mol.Cell.Biol.*11,2296-2302
10. Stewart,C.J.,Ito,M.,and Conrad,S.E. (1987) Mol.Cell.Biol.7,1156-1163
11. Artishevsky,A.,Deleagene,A.M.,and Lee,A.S. (1984)*Mol.Cell.Biol.* 4,2364-2369
12. Manley,J.L.,Fire,A., Cano, A., Sharp, P.A., and Gefter,M. L. (1980) Proc. Natl.Acad.Sci. U.S.A. 77,3855-3859
13. Dou, Q.P.,Fridovich-Keil,J.L.,and Pardee,A.B. (1991) Proc. Natl. Acad. Sci. U.S.A. 88,1157-1161
14. Bradley,D.W.,Dou,Q.-P.,Fridovich-Keil,J.L.,and Pardee,A.B. (1990) Proc.Natl.Acad.Sci. U.S. A.87,9310-9314
15. LaBella, F., Gallinari,P., McKinney, J., and Heintz,N. (1989) *Genes and Dev.* 3, 1982-1990
16. Osley,M.A. (1991) Ann.Rev.Biochem. 60, 827-861