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Identification of a G_1-S-Phase-Regulated Region in the Human Thymidine Kinase Gene Promoter

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We have identified a regulatory region in the human thymidine kinase gene promoter. A set of promoter deletion mutants was constructed, linked to the bacterial neomycin resistance gene, and stably transfected into Rat3 cells. It was shown that the region between 135 and 67 base pairs upstream of the cap site is required for conveying G_1-S-phase regulation to the linked *neo* gene. In addition, primer extension assays demonstrated that the same transcriptional start sites were used in G_1- and S-phase cells and in the various deletion mutants tested.

Thymidine kinase (TK; EC 2.7.1.21), is a relatively widespread enzyme that is present in nearly all organisms. TK activity is highly regulated by both the stage of the cell cycle and the growth state of the cell. Terminally differentiated and nonproliferating cells express very little TK activity (12), but growing cells express high levels of the enzyme. When quiescent, serum-starved cells are induced to reenter the cell cycle by the addition of fresh serum, enzyme levels remain low throughout G_1, increase sharply at the G_1-S boundary, and remain elevated throughout S and G_2 (6). A similar pattern of enzyme regulation is seen in continuously cycling cells (13), indicating that at the level of the protein product this gene is truly cell cycle and not simply growth regulated. In contrast to TK enzyme and protein levels, TK mRNA appears to be regulated differently in continuously cycling and serum-stimulated cells. In the case of serum-stimulated cells, the enzyme activity is paralleled by the steady-state levels of TK mRNA, which also remain low during G_0 and G_1 and increase approximately 10- to 20-fold as cells enter S phase (2, 15, 16). In continuously cycling cells, only a two- to threefold difference in TK mRNA levels between G_1 and S-G_2 phases is detected (13).

The function of the human TK promoter has been investigated by several groups. By assaying deletion mutants for abilities to transfect TK^- cells to a TK^+ phenotype, Kreidberg and Kelly defined the minimal promoter to within 83 base pairs (bp) upstream of the transcriptional start site and showed that a fragment containing only 53 bp of upstream sequences had no promoter activity (9). A similar deletion analysis of the TK promoter region was done by Arcot et al. (1), but the promoter deletions were linked to the bacterial chloramphenicol acetyltransferase (*cat*) gene and assayed in transiently transfected mouse L cells. These experiments showed a gradual decrease in promoter strength as 5′ sequences were deleted, with a construct containing 139 bp upstream of the mRNA cap site still possessing 64% of the promoter activity. The next two fragments tested, which contained 88 and 58 bp upstream of the cap site, possessed only 28 and 19% activity, respectively.

Several lines of evidence indicate that the TK gene promoter is transcriptionally regulated, at least in serum-stimulated cells. First, by using nuclear run-on transcription assays, we and others have shown that transcription of the gene increases 2- to 11-fold at the G_1-S boundary in serum-stimulated cells (2, 10, 15). Second, several groups have shown that a fragment containing approximately 400 bp upstream of the cap site is sufficient to confer G_1-S-phase regulation to linked heterologous genes, such as the bacterial *cat* (18) or *neo* (7) gene. A second fragment containing only 67 bp upstream of the mRNA cap site gives rise to a constitutively low level of expression in transfected cells (7). Experiments from several laboratories using TK cDNA clones expressed from heterologous promoters indicate that sequences within the body of the TK cDNA may also play a role in the regulation of TK mRNA levels, making the pattern of expression independent of the promoter used (10, 16). Recent results with additional promoters and more extensive time courses, however, have indicated that the promoter does play a major role in the pattern of expression (5, 11). Since in our experiments (5) we saw some effect of TK cDNA sequences when they were expressed from the herpes TK promoter, we have chosen to study the TK promoter linked to a heterologous *neo* gene.

In order to more precisely define the region of the TK promoter responsible for the G_1-S-phase activation of the gene in serum-stimulated cells, we examined the sequences that are required for both basal expression and regulation of the gene. A series of human TK promoter deletions containing various amounts of 5′-flanking sequences was constructed by digestion with convenient restriction endonucleases (*Eco*RI, *Pst*I, *Acc*I, *Dde*I, *Hin*fI, or *Nco*I) at the 5′ ends and *Sca*I (located 30 bp downstream of the cap site) at the 3′ end (Fig. 1). *Hin*dIII linkers were ligated to both ends, and the resulting promoter fragments were cloned into a *Hin*dIII site upstream of the bacterial *neo* gene (14) in a pUC19 vector. The resulting constructs contained a simian virus 40 polyadenylation site and a small T-splice donor-acceptor at the 3′ ends. Plasmids containing the hybrid genes were transfected into Rat3 cells (17), as described by Wigler et al. (19), and G418-resistant colonies were selected in medium containing 800 μg of G418 per ml. When the transfection efficiencies of these constructs were compared by using 100, 200, and 400 ng of DNA per 10-cm plate, the constructs were indistinguishable, with the exception of 67-Neo, which gave rise to four to five times fewer colonies (data not shown). Since the number of colonies did not increase linearly with the amount of DNA used in this experiment, we repeated the experiment with 444-Neo,

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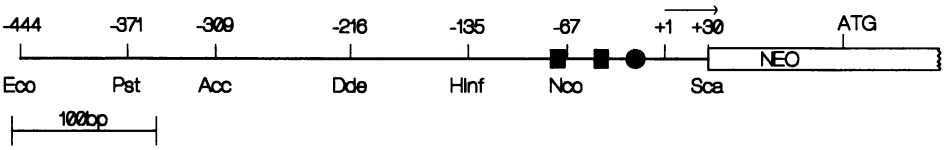

FIG. 1. Structure of human TK promoter-*neo* hybrid gene constructs. The restriction enzyme sites which were used to construct the deletion mutants and their positions relative to the transcriptional start site (+1) are shown. The constructs were named according to the location of the restriction enzyme used at the 5′ end of the human TK promoter, e.g., an *Eco*RI site located at 444 bp upstream of the cap site was used to generate 444-Neo. Symbols: ●, putative TATA box; ■, putative inverted CCAAT boxes.

135-Neo, and 67-Neo and smaller amounts of DNA (5, 25, 50, and 100 ng/10-cm plate). 444-Neo and 135-Neo transfected with approximately the same efficiencies over this range of DNA concentrations, but 67-Neo transfected with 5- to 11-fold less efficiency (Table 1). The transfection frequency in this experiment was dose dependent for all constructs.

To investigate the regulation of these hybrid genes, we first determined whether 444-Neo showed the same pattern of expression as human TK minigene constructs. Pools of 30 to 300 G418-resistant Rat3 colonies containing either 444-Neo or a TK minigene construct containing the same promoter fragment linked to the human TK cDNA (444-TK) were grown to confluence, serum starved in 0.5% serum for 24 h, and then serum stimulated by the addition of fresh medium containing 10% serum. Total-cell RNA was prepared at various times after serum stimulation by the method of Favaloro et al. (4), and 20 μg of RNA from each time point was analyzed by Northern (RNA) blotting, as described by Stuart et al. (16). *neo* mRNA levels were low at 0 and 3 h, increased by 8 h, and reached their highest levels at 12 h (444-Neo, Fig. 2A). The relative amounts of *neo* mRNA were quantitated by scanning the autoradiograms with an LKB UltroScan XL laser densitometer, and induction values were calculated by dividing mRNA levels at 12 h by the levels at 0 h. The results of these experiments revealed that the increase of *neo* mRNA between 0 and 12 h was approximately ninefold. This pattern of expression was virtually indistinguishable from the pattern seen with the TK minigene (Fig. 2B), in which expression increased 17-fold between 0 and 12 h. The reason for the difference between 9- and 17-fold increases is unknown, but it may be due to posttranscriptional regulation mediated by the body of the human TK gene.

After we confirmed that 444-Neo was regulated in the same way as a human TK minigene, the remaining promoter deletion mutants were investigated (Fig. 2A). These experiments were repeated three times with three independent pools of cells (except in the case of 67-Neo, for which the experiment was repeated twice with two pools of cells). The pattern of *neo* expression was not affected by the deletion of 5′ sequences up to −135, since the induction of mRNA increased between 0 and 12 h with 371-neo, (fourfold), 309-Neo, (fivefold), 216-Neo (eightfold), and 135-Neo (sevenfold). Similarly, a 19-fold increase was seen with a human TK minigene (135-TK) expressed from the 135-bp promoter (Fig. 2B). The deletion mutant 67-Neo had lost the ability to

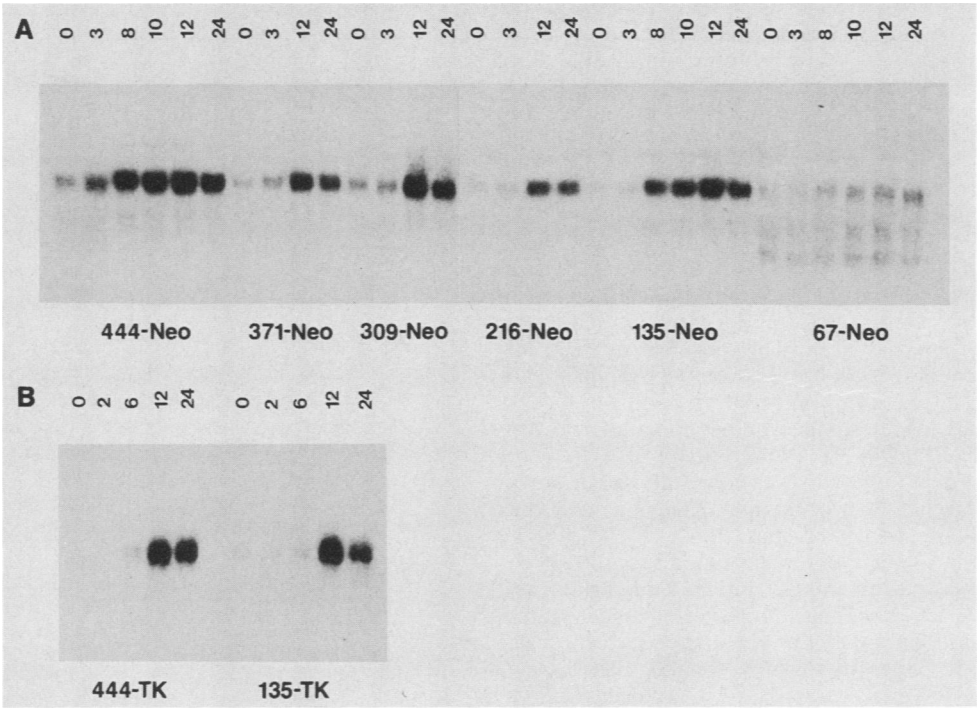

FIG. 2. Northern blot analysis of human TK promoter-*neo* gene (A) and TK minigene (B) constructs in pools of stably transfected Rat3 cells. Total RNA was extracted at the indicated times (hours) after serum stimulation, and 20 μg of RNA per lane was analyzed.

TABLE 1. Transfection efficiencies of human TK promoter-*neo* constructs in Rat3 cells[a]

Construct	No. of G418-resistant colonies at DNA concn (ng/plate) of:			
	5	25	50	100
444-Neo	9	33	97	165
135-Neo	6	55	70	131
67-Neo	1	3	11	34

[a] Transfections were carried out as described by Wigler et al. (19). Each number is the average of two plates. A negative control with carrier DNA alone yielded no colonies.

be induced at the G_1-S interface, showing only a 1.2-fold induction by 12 h.

We also wished to determine if the absolute levels of mRNA expression varied with the different constructs, as was suggested by the transient-expression *cat* assay data of Arcot et al. (1). Gels such as those shown in Fig. 2A were scanned, and the levels of mRNA produced by the different constructs were compared at 0, 3, and 12 h (data not shown). The results of this analysis (an average of three experiments) indicated that the level of expression from all promoters including the 67-Neo construct varied by at most twofold at 0 h and throughout G_1. The same was true at 12 h, except for 67-Neo, which expressed four- to fivefold less RNA than the other constructs. These results suggest that the 67-bp promoter fragment functions as well as do the other fragments during G_0 and G_1 but fails to be induced as the cells enter S phase.

We have addressed the question of whether G418 selection affects either the overall level of expression or the regulation of the hybrid genes. For example, the truncated promoter constructs may lower levels of expression, but colonies expressing low levels of the product may be selected against. Except in the case of 67-Neo, this seems unlikely, since all constructs had indistinguishable transfection efficiencies (Table 1). A second possibility is that the selection protocol selects against transfectants that efficiently shut off the *neo* gene as cells become confluent. To test these possibilities, 444-Neo was cotransfected with a plasmid containing the human TK cDNA expressed from the simian virus 40 early promoter (SV-huΔScaTK) (5), and stable transfectants were selected in medium containing either hypoxanthine-aminopterin-thymidine (HAT) or G418. The levels and pattern of *neo* mRNA expression were examined in pools of cells selected by both methods. These experiments showed a fivefold increase between 0 and 12 h for the colonies selected in G418 and a threefold induction when HAT selection was used. This experiment indicates that the expression of the 444-bp TK promoter fragment was not significantly influenced by selection in G418.

We also wished to determine whether the same mRNA cap site was utilized in G_0-, G_1-, and S-phase cells with the various promoter deletions. This was especially important in the case of 67-Neo. The transfection efficiency with this construct was 5- to 11-fold less than those of the others, which suggests that the site of integration affects the ability of the gene to be expressed and that integrated hybrid genes may be expressed from a cellular promoter-enhancer with a different transcriptional start site. In addition to the full-length *neo*-specific bands in Fig. 2A, smaller bands were seen in Northern blot analyses with 67-Neo, which suggests that there are *neo* transcripts that are truncated at either their 5′ or 3′ ends.

We examined the transcriptional start sites of 444-Neo, 135-Neo, and 67-Neo in G_0, G_1, and S phases by using the primer extension assay described by Eisenberg et al. (3). The primer was a 25-base oligonucleotide (5′-CGCACTGGCT TTCTACGTGTTCCGC-3′) which hybridizes between 72 and 47 bp downstream of the *Hin*dIII site at the 5′ end of the *neo* gene. The extended products are predicted to be 101 and 95 nucleotides long if both TK cap sites identified by Kreidberg and Kelly are used (9). All of the human TK-*neo* constructs tested gave rise to products of the expected length at 0 and 12 h (Fig. 3), which indicates that the same start sites are used in G_0- and S-phase cells. In addition, the results demonstrate that expression from the truncated 67-bp promoter was in fact initiated at the TK cap site and not from cryptic upstream promoter sequences contributed by the plasmid or cellular DNA. Densitometer scanning revealed 8-, 15-, and 2-fold increases with 444-Neo, 135-Neo, and 67-Neo, respectively. A second independent primer extension experiment with different pools of cells at 3 h (G_1) and 12 h (S) showed six-, nine-, and twofold increases with 444-Neo, 135-Neo, and 67-Neo, respectively. The increase was similar to the results obtained from densitometer scannings of Northern blots (Fig. 2A).

In the experiments reported here, we used a set of TK promoter deletions linked to the bacterial *neo* gene to study both levels and patterns of *neo* gene expression in stably transfected Rat3 cells. Our results confirm earlier studies, which show that the TK promoter is sufficient to confer serum regulation to linked heterologous genes and further indicate that sequences between −67 and −135 bp upstream of the cap site are required for the specific G_1-S-phase regulation of the promoter in serum-stimulated Rat3 cells. Since the level of expression of the 67-Neo construct was the same as levels for the other constructs in G_0- and G_1-phase

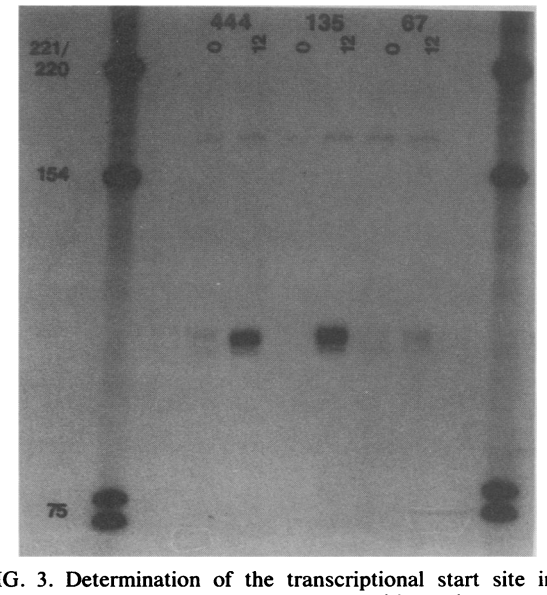

FIG. 3. Determination of the transcriptional start site in the human TK promoter-*neo* gene constructs with a primer extension assay. Total RNA was extracted at the indicated times (hours) after serum stimulation, and 20 μg of RNA per extension reaction was analyzed. *Hin*fI-digested pBR322 was labeled at its 5′ end with [γ-^{32}P]-ATP and used as a size marker (lanes M, in base pairs). The strands of the 75-bp fragment separate under the conditions used and generated two bands. An additional band of approximately 190 bp is seen in every lane. It appears not to be cell cycle regulated and is of unknown origin.

cells and since the same transcriptional start site was used with this and with the other promoters, we believe that the element between −67 and −135 is a regulatory, not a basal, transcription element. A caveat to this interpretation is that 67-Neo transfects with 5- to 11-fold less efficiency than the remaining constructs, so we might have selected for cells with high basal levels of expression. Since the 67-Neo construct is still slightly (twofold) induced in S-phase cells, it is possible that part of this element or another element is located downstream of −67. To definitively show that this region contains a regulatory element distinct from the basal promoter, it will be necessary to construct and test specific point mutations or to link the putative regulatory element to another, nonregulated promoter.

We propose that the G_1-S-phase-specific induction of *neo* mRNA in our experiments is due to an increase in the level of transcription of the TK-*neo* hybrid genes. Since the constructs used in this study also contain 30 bp of the 5′ untranslated region of the cDNA and since several studies have suggested that TK mRNA levels are regulated posttranscriptionally (2, 10, 15), it could be argued that some or all of the regulation seen is a result of posttranscriptional events. We believe that this explanation is unlikely, since 67-Neo also contains the same 30 bp but is not regulated to the same extent as the other constructs. It is possible, however, that the residual regulation of 67-Neo is due to posttranscriptional regulation mediated by these sequences. In order to resolve this issue, we performed nuclear run-on transcription assays of Rat3 cells containing 444-Neo, using the endogenous glyceraldehyde-3-phosphate dehydrogenase gene as a nonregulated control. These experiments showed an approximately 3- to 4-fold increase in the rate of transcription of 444-Neo at 10 h following serum stimulation over the rate at 0 h (G_0) and a 5- to 10-fold increase over the rate at 4 h (G_1) (data not shown).

While the manuscript was in review, others reported that the region between −83 and −64 bp is important for S-phase-specific regulation of TK mRNA levels in Syrian hamster fibroblasts (11). Potential regulatory sequences within this region include one of two inverted putative CCAAT boxes. Both CCAAT boxes have been investigated in protein-binding studies (1, 8) and have been shown to bind factors. A description of the role of sequences in this region in regulation of the gene awaits a more detailed analysis.

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