Inducible proteins binding to the murine thymidine kinase promoter in late G1/S phase

(cell-cycle regulation/DNA binding proteins/Sp1)

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ABSTRACT By performing DNase I footprint and bandshift analyses of a 170-base-pair region of the murine thymidine kinase promoter, we identified an inducible DNA binding activity that we named Yi. Yi binding activity was not detected in G0 and G1 extracts, but it was observed as cells crossed the G1/S boundary. Yi proteins bind specifically to a consensus sequence (CCCNCNNNCT) found at three distinct sites in this promoter region. We also observed a murine Sp1 binding activity that was constitutive throughout the cell cycle. We propose that the G1/S-specific Yi binding is important for murine thymidine kinase gene regulation and perhaps also for initiation of DNA synthesis.

Studies of thymidine kinase (TK) expression provide a good model for investigating the mechanisms by which genes are regulated during the cell cycle. Expression of the TK gene is regulated with respect to cell proliferation at the levels of transcription, posttranscription, and translation (1–11). Our purpose for studying the regulation of TK gene expression is to understand coordinate control of events at S-phase onset (12). The tight coupling of the induction of TK gene expression with the onset of S phase (DNA synthesis) suggests that some prior common control mechanism may regulate both events, either directly or indirectly.

The upstream regulatory region of the murine TK gene (13, 14) does not have either CCAAT or TATA consensus elements, and it is markedly different from that of the human TK gene (15, 16). Lieberman et al. (13) reported that a 291-base-pair (bp) DNA fragment from the 5′ end of the murine TK gene has promoter activity. Recently, we found that sequences located between −174 and +159 bp of the TK translation initiation site are sufficient to confer efficient S-phase-specific regulation to the expression of two different reporter genes—chloramphenicol acetyltransferase and β-globin (28).

In the studies reported here, we have identified three protein binding regions—MT1 (−104 bp/−84 bp), MT2 (−83 bp/−67 bp), and MT3 (−43 bp/−28 bp) (MT, murine TK)—within the murine TK 170-bp upstream sequence. These positions are given relative to the TK translational start site to avoid confusion caused by the multiplicity of transcriptional start sites in the murine TK gene (J. M. Gudas, M. W. Datta, J. L. F.-K., and A. B. P., unpublished data). MT1 includes a perfect Sp1 consensus binding site (GGGCCTG) and binds a murine Sp1-like protein, which we refer to as murine Sp1 although the degree of homology between this protein and human Sp1 remains to be determined. Murine Sp1 was the first DNA binding protein we identified in this system. By using DNase I footprint and bandshift assays, we have identified a second, cycle-regulated binding activity that we named Yi. The third binding activity was named Bing. (Yi and Bing are the second and third signs, respectively, in the Chinese zodiac).

MATERIALS AND METHODS

Cell Culture and Synchronization. BALB/c 3T3 clone A31 cells were grown as monolayer cultures as described (5). Cells were harvested at near confluence.

Cell synchronizations were performed as described (28). Briefly, cells were plated at 5 × 105 cells per plate onto 150-mm tissue culture dishes and grown for 3 days. Cultures were then washed twice with low serum Dulbecco’s modified Eagle’s medium (0.4% bovine calf serum) and incubated in this medium for 60 hr. The medium was removed and the cells were refed 10% serum-containing medium and harvested at the indicated times. Entry of cells into S-phase was monitored by incorporation of [3H]thymidine essentially as described (17).

Synthetic Oligonucleotides. The oligonucleotides containing a MT sequence (MT1, 21 bp at −104 to −84 bp; MT2, 17 bp at −83 to −67 bp; MT3, 21 bp at −43 to −23 bp), a related sequence, or the AP1 binding site (13 bp; for its sequence, see Fig. 1C) with Xho I-compatible ends were synthesized on an Applied Biosystems model 308 synthesizer at the Dana-Farber Oligonucleotide Facility. Complementary synthetic oligonucleotides were annealed in 67 mM Tris buffer (pH 7.5) containing 13 mM MgCl2, 6.7 mM dithiothreitol, 1.3 mM spermidine, and 1.3 mM EDTA (18). Oligonucleotides used for bandshift analyses were end-labeled with [γ-32P]dNTP and Klenow enzyme.

Nuclear Extract Preparation, DNase I Footprint, and Bandshift Analyses. Nuclear protein extracts were prepared as described by Dignam et al. (19). Footprint assays were performed with an adaptation of a method described by Lee et al. (20). Bandsift assays were performed essentially as described by Fried and Crothers (21) and by Knight et al. (4).

RESULTS

The Murine TK 5′ Sequence −174 to −4 bp Contains Three Distinct Protein Binding Regions. We have found that sequences located between −174 and +159 bp of the murine TK translational start site are sufficient to drive efficient S-phase-specific serum-responsive expression of both chloramphenicol acetyltransferase and β-globin reporter genes in transfected mouse fibroblasts (28). To further define murine TK promoter regulatory elements by independent methods, we have investigated DNA sequences within the TK promoter that are bound by murine nuclear proteins in vitro. Using DNase I footprint analysis with crude nuclear extracts prepared from exponential murine A31 cells, we identified three protected regions on both coding (Fig. 1A, lane 3) and

Abbreviation: TK, thymidine kinase.

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noncoding (see Fig. 5; data not shown) strands; we named these regions MT1 (~104 bp/−84 bp), MT2 (~83 bp/−67 bp), and MT3 (~43 bp/−28 bp), (Fig. 1B). We observed less protection on MT2 than on either MT1 or MT3, suggesting that the proteins that interact with MT2 may bind their target site(s) with relatively weaker affinity. This observation is consistent with the results of bandshift and cross-competition bandshift experiments (see Fig. 3; data not shown).

MT1 Includes an Sp1 Binding Site. The MT1 binding region includes a perfect GC box (~96 GGGCGGGGG −88; Fig. 1B), which is a binding site for the transcription factor Sp1 (22). To determine whether a murine protein similar to the human transcription factor Sp1 is involved in the protection of this GC box region, we performed DNase I footprint analysis with highly purified human Sp1 protein (kindly provided by S.P. Jackson and R. Tjian, Department of Biochemistry, University of California at Berkeley). Because nearly all of the MT1 region was protected by the purified Sp1 protein (Fig. 1A, lane 4; data on noncoding strand not shown), it is likely that this G+C-rich region is a murine Sp1 binding site. The MT2 and MT3 elements were not protected from digestion by the purified Sp1 protein, consistent with the assertion that they are independent binding regions.

To test the functional significance of Sp1 binding at MT1, a point mutation (changing a G residue to a C at −93 bp) was introduced into the MT1 region, virtually eliminating expression of a chloramphenicol acetyltransferase reporter gene in transfection assays (28). The analogous point mutation in a human Sp1 consensus site reduced Sp1 binding by >30-fold relative to binding at the wild-type site (23). We performed DNase I footprint and bandshift analyses of the wild-type and mutant murine MT1 sequences. Footprint analysis with purified human Sp1 protein indicated that the point mutation in the MT1 region prevented most, if not all, Sp1 binding (data not shown). Fig. 2 illustrates a bandshift assay using 21-bp synthetic double-stranded oligonucleotides representing the wild-type and mutated MT1 elements, with crude nuclear extract prepared from mouse A31 cells. Three major complexes were observed when the wild-type MT1 oligonucleotide was used (lanes 3 and 5). The top two bands (indicated as Sp1) were eliminated by introduction of the Sp1 point mutation (lanes 4 and 6), suggesting that these complexes involved a murine Sp1 protein. This conclusion was further supported by Southern blot analysis of A31 nuclear

![Footprint analysis of murine TK 5' sequence](image)

**Fig. 1.** Footprint analysis of murine TK 5' sequence. The DNA fragments used in this experiment were isolated from the plasmid pCATm(−174)B (J.L.F.-K. and A.B.P., unpublished work). The coding strand was labeled at the BamHI site and then cut with XhoI to release a 170-bp fragment. The DNA fragments were incubated with A31 nuclear extracts or purified Sp1 protein and then digested with DNase I. Lanes: 1, G+A Maxam–Gilbert sequencing ladder (positions indicated); 2, DNase-treated fragment without nuclear extract (control); 3, DNase-treated fragment with 115 μg of nuclear extract; 4, DNase-treated fragment with 10 ng of purified Sp1 protein (Sp1). Protected regions are indicated. (B) Summary of DNase I footprint data. The sequence of the upstream promoter region of the murine TK gene is shown (13, 14). Brackets indicate protected sequences—designated MT1, MT2, and MT3. Yi consensus sequences CCCCNCCNCT are denoted by solid lines, and the Sp1-binding site is indicated by a dashed line. The TK translational start site, ATG, is boxed. (C) Sequence of API oligonucleotide used as a competitor in experiments described in Fig. 3.
Oligonucleotide

...two polypeptides with apparent molecular masses of 105 and 92 kDa, similar to those reported for human Sp1 (105 and 95 kDa) (24), were found to bind specifically to a labeled wild-type MT1 oligonucleotide (data not shown). The Sp1 point mutation had little effect on the formation of the lower complex (compare lanes 4 vs. 3 and 6 vs. 5). Additional evidence suggests that this lower band was derived from a distinct protein complex, rather than from a single protein, that we named Yi (Figs. 3 and 4; data not shown).

Yi Protein Complex Has an Apparent Consensus DNA Binding Sequence. When the results from footprint analyses were summarized, we noted that all of the protected regions were G+C-rich (Fig. 1B). Carefully comparing them, we identified a consensus sequence CCCCNCCNCT in all three MT elements (underlined in Fig. 1B). We propose that a protein complex, Yi, interacts with the three MT sequences. This model is consistent with the results presented in Fig. 2, which demonstrate that, in addition to murine Sp1, another set of nuclear proteins, Yi, recognize and bind to the MT1 element. This Yi binding activity was apparently unaffected by the Sp1 point mutation (located in the first N in the consensus CCCCNCCNCT). We postulate that the Yi and murine Sp1 binding sites in MT1 overlap.

To test this hypothesis, we performed competition experiments by using bandshift assays with synthetic double-stranded oligonucleotides containing MT1, MT2, and MT3 sequences. As a negative control, we also used a synthetic probe that included an AP1 binding site. Crude nuclear extract from A31 cells was incubated with labeled MT1 sequence in the absence or presence of various unlabeled competitors. Fig. 3 illustrates that both Sp1 and Yi binding to the labeled MT1 sequence were blocked by self-competition with a 10- or 60-fold molar excess of unlabeled MT1 DNA (compare lanes 3 and 4 vs. lanes 2 and 12), while only the Yi band, not the Sp1 bands, was blocked by competition with equal amounts of MT3 or MT2 sequences (lanes 5–8). Neither Sp1 nor Yi binding was blocked by competition with a synthetic oligonucleotide containing an AP1 binding site at 10-, 60-, or even 140-fold molar excess (lanes 9–11), indicating the relative specificity of Yi binding. Analogous experiments with labeled MT2 or MT3 oligonucleotides gave similar results (data not shown).

Yi Binding Activity Is Induced at the G1/S Boundary. The identification of three protein binding elements within the upstream region of the murine TK promoter sequence (Fig. 1) raises the possibility that some of these elements may contribute to the cell-cycle regulation of murine TK gene expression. We therefore investigated in vitro nuclear protein binding to these three elements by using bandshift assays with nuclear extracts prepared from synchronized populations of cells (Fig. 4). Briefly, A31 cells were synchronized in quiescence by serum starvation and then restimulated to proliferate by the addition of fresh serum; crude nuclear extracts were prepared at the indicated times after serum stimulation. The data in Fig. 4 demonstrate that by 12 hr after serum stimulation Yi protein complex bound to labeled oligonucleotides containing the MT3 (Fig. 4A), or MT1 (Fig. 4B) sequences. When labeled MT3 oligonucleotide was used (Fig. 4A), the Yi-DNA complex (as confirmed by bandshift competitions; data not shown) was undetectable in G0 cells (0 hr), was detected only at very low levels in G1 phase cells (1, 4, and 8 hr), and increased sharply as cells crossed the G1/S boundary (12 hr). This Yi binding activity remained elevated throughout the S and G2 phases (16 and 20 hr). Also, a transiently induced protein binding activity, marked TKE (TK early protein binding), was observed 1 hr after serum stimulation of the G0 cells.
A similar cell cycle study was performed with a labeled MT1 oligonucleotide (Fig. 4B). As expected, we observed two types of binding activities. The top two bands (labeled Sp1), which we attribute to the binding of mouse Sp1 protein (as shown in Fig. 2), were unchanged throughout the G0 to S transition. These bands therefore provide a convenient internal control, indicating that equal amounts of nuclear extract were loaded in each lane. In contrast, the lower band visible in this gel, which we attribute to Yi binding (Figs. 2 and 3), demonstrated a dramatic G1/S-phase-specific induction similar to that seen in MT3.

We also observed that a small percentage of MT2 oligonucleotide was shifted by nuclear extracts. With a longer exposure of the film, we were able to detect the same G1/S phase-specific Yi binding to MT2 as well as another constitutive binding, which we attribute to another protein that we have named Bing (data not shown).

Since both the murine Sp1 and Bing proteins bind constitutively to their target sequences located in MT1 and MT2, respectively, we predicted that only the MT3 binding region should show significant cell-cycle-specific changes in protection patterns revealed by DNase I footprint analysis. To test this prediction, we performed footprint analyses with nuclear extracts made from G0 (0 hr), G1/S (12 hr), and S/G2 (20 hr)-phase cells. The data presented in Fig. 5 demonstrate that both MT1 and MT2 sequences were protected at all three times. In contrast, protection of MT3 was not found when G0 extract was used but was observed in both G1/S and S/G2 extracts (lanes 2-4). We observed stronger Yi protection of the MT3 element on the noncoding strand than on the coding strand (data not shown). These results further support the conclusion that Yi binding activity is serum induced in a G1/S-phase-specific manner.

**DISCUSSION**

To investigate protein binding sites located within a 170-bp region of the murine TK promoter, we have performed footprint analyses and identified three binding elements—MT1, MT2, and MT3 (Fig. 1). By using bandshift and footprint analyses with nuclear extracts prepared from cells harvested at different times after serum stimulation from quiescence, we have identified a protein complex from S-phase but not from G0-phase cells. This complex, which we have named Yi, binds in late G1/S to a consensus sequence located in these three MT regions of the TK promoter (Figs. 4 and 5).

It is important to note that the point mutation in the Sp1 site of MT1 (located in the first N in the Yi consensus sequence) reduced Sp1 binding but had little apparent effect on Yi binding (Fig. 2). The relative specificity of Yi binding was demonstrated by bandshift competition experiments. Yi binding to any labeled MT oligonucleotide was blocked by competition with any unlabeled oligonucleotide containing the consensus sequence, but not by an API binding sequence, which lacks the consensus sequence (Fig. 3; data not shown). Finally, we made several base substitution mutations in the consensus sequence in MT3, which reduced Yi binding to this element to various extents (data not shown). This 10-bp Yi consensus binding site has 60% of the sequences fixed, which is comparable to the percentage of fixed bases (GGGNNTNNCC) in the NF-kB consensus sequence (25). MT2 appears to bind Yi less strongly than either MT1 or MT3 (Figs. 1 and 5). One possible interpretation is that orientation of the consensus sequence CCCCNNNNCT may affect binding; the MT2 consensus sequence is inverted relative to those in MT1 and MT3 (Fig. 1B).
The level of Yi binding activity was low throughout G0 and G1 phases, increased sharply at the G1/S boundary, and remained elevated throughout S and G2 phases (Fig. 4), correlating well with the G1/S-phase-specific induction of TK gene expression. These kinetic data suggest that the Y protein complex may regulate expression of the murine TK gene. Preliminary mutagenesis studies support this possibility and indicate that MT3 is necessary for proper S-phase-specific regulation of a β-globin reporter gene expressed from the murine TK promoter (J.L.F.-K., Q.-P.D., and A.B.P., unpublished work).

Yi binding activity is relatively constitutive throughout the cell cycle in transformed BPA31 cells, in contrast to the G1/S-phase-specific increase in Yi binding activity seen in nontransformed A31 cells. Furthermore, Yi binding activity is unstable in A31 cells, but it is stabilized in BPA31 cells treated with cycloheximide (26). This Yi binding activity thus has properties identical to those predicted for a candidate regulatory protein influencing the initiation of DNA synthesis (27). These data raise the possibility that Yi binding activity may influence not only the expression of the murine TK gene but also the initiation of DNA synthesis in normal cells.

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