Sp1 and NF-Y Are Necessary and Sufficient for Growth-dependent Regulation of the Hamster Thymidine Kinase Promoter*

(Received for publication, March 1, 1999, and in revised form, July 8, 1999)

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Thymidine kinase (TK) genes from different species are growth- and cell cycle-regulated in a very similar manner; still, the promoter regions of these genes show little homology to each other. It was previously shown that the murine TK gene is growth-regulated by Sp1 and E2F. Here we have characterized cis-regulatory elements in the hamster promoter that are essential and sufficient to confer efficient and serum-responsive expression. The TK promoter was isolated from baby hamster kidney cells. DNase I protection experiments revealed a protected region from positions 24 to 99 relative to the transcription start site. Within this region, binding sites for the transcription factor Sp1 and a CCAAT box, which interacts with the transcription factor NF-Y, were identified. An E2F-like sequence was found not to bind protein, and its removal did not affect promoter activity. This was supported by the observation that cotransfection of a hamster TK reporter gene construct with E2F-1 does not lead to transactivation of the promoter. A 122-base pair region that contains a single Sp1 site, a CCAAT box, and a TATA element was found to be sufficient for serum-responsive expression of a reporter gene. Mutations that inactivate any one of these three elements caused a strong reduction or a loss of promoter activity.

Thymidine kinase (TK)1 is a salvage pathway enzyme that functions in the phosphorylation of thymidine to form TMP at the expense of ATP and thereby contributes to the pool of thymidine nucleotides for DNA replication. Mammalian cells carry two TK genes, one coding for the mitochondrial enzyme and the other one for the cytoplasmic enzyme, which is the subject of this report. The expression of cytoplasmic TK is strongly growth- and cell cycle-regulated at the transcriptional as well as at several post-transcriptional levels (reviewed in Refs. 1 and 2). TK mRNA is hardly detectable in growth-arrested cells, whereas its synthesis is dramatically stimulated at the border of G1 to S phase of the cell cycle. Many genes coding for enzymes involved in DNA replication and precursor production are regulated similarly. Not surprisingly, the promoters of such genes exhibit high degrees of sequence homology between organisms. For instance, the promoter of another gene involved in precursor production, that of dihydrofolate reductase, is very similar between human and various different rodents (reviewed in Ref. 3). This is not true for the TK genes, where different species show remarkably divergent sequences in the promoter region, whereas the expressed part of the genes is highly homologous. This might indicate variation in the mechanisms of transcriptional regulation of these genes. The TATA-less murine TK promoter (4), for example, contains one Sp1-binding site and a binding motif for the growth-regulated transcription factor family E2F within the region important for growth-regulated expression (5, 6), the latter closely resembling a similar motif present in the promoter of the dihydrofolate reductase gene. Both the Sp1 and E2F sites are necessary for regulated expression of the murine TK gene (5–7), and Sp1 and E2F-1–3 have in fact been shown to directly interact (8). The human TK promoter, on the other hand, carries Sp1 sites, several motifs resembling E2F-binding sites, two reversed CCAAT boxes, and a TATA element (9). The upstream region of the TK gene from Chinese hamster cells was reported previously (10–12); however, transcription factor-binding sites and the proteins involved in the regulation of the gene have not been identified. We were intrigued by the differences in the structures of the TK promoters, which contrast with the high homology displayed by the expressed part of the genes, and report here on our detailed analysis of the hamster TK promoter derived from BHK cells. We present evidence that this TATA-containing promoter is regulated by Sp1 and the CCAAT-binding transcription factor NF-Y, whereas the E2F-like sequence is dispensable and does not bind transcription factors of the E2F family. The transcription factor pair Sp1/NF-Y is active on the hamster promoter and appears to functionally replace the transcription factor pair Sp1/E2F, which is active on the murine TK promoter. Thus, although the murine and hamster TK genes are regulated very similarly during growth, the mechanism of this regulation is clearly distinct. Furthermore, from the results presented in this report, it appears questionable to generally regard TK as an E2F-regulated gene.

EXPERIMENTAL PROCEDURES

Isolation of the Hamster TK Promoter from BHK Cells and Plasmid Constructions—Genomic DNA was prepared from BHK cells using standard procedures (13). The TK promoter was amplified by polymerase chain reaction using primer sequences derived from the upstream region (5′-CAACGGGTACCTGCGCTGGA-3′) and from exon 1 (5′-GTATTGCTACGGCGCCGTCC-3′) of the Chinese hamster TK gene (11). The polymerase chain reaction products contained engineered restriction sites for KpnI and NheI, which were used for subcloning into pGem3zf(-). Four different clones were sequenced to avoid polymerase chain reaction-generated mutations.

Mutations within the promoter region were produced using the BioRad system for oligonucleotide-directed in vitro mutagenesis. The mutations were chosen to produce a unique restriction site at the selected position and are schematically presented in Fig. 5C. All mutations were
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verified by sequencing. Wild-type and mutated TK promoter fragments were cloned into the KpnI and NheI sites of the plasmid pGL2neo. This plasmid was constructed by introducing the neo gene under the control of the SV40 early promoter into the BamHI site of plasmid pGL2-Basic (Promega).

For the construction of truncated promoter fragments, the unique restriction sites in the mutated transcription factor-binding sites were used. The fragments were cut at these sites; blunt-ended; and then digested with NheI, followed by cloning into correspondingly digested pGL2neo.

Cell Culture, Transfection, and Selection of a Stable Cell Line—BHK cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin, and streptomycin. To generate stably transfected cells, 5 μg of the plasmid of interest were linearized with XmnI and transfected into BHK cells using Polybrene-assisted gene transfer (14). Stably transfected cells were selected in the presence of Geneticin (600 μg/ml; Life Technologies, Inc.). Clones were pooled after 12–14 days and expanded in medium containing Geneticin (300 μg/ml). The drug was removed only shortly prior to the experimental assays.

Harvesting of cells and preparation of cell extracts for luciferase assays were performed as described elsewhere (15). Growth arrest of cells in G0 was accomplished by reducing the concentration of serum to 0.5% for 72 h. Restimulation was achieved by the addition of serum to 20%.

For flow cytometry, cells were trypsinized, washed with phosphate-buffered saline, and fixed in 85% ethanol. The fixed cells were suspended in a few drops of 0.05% pepsin, stained with 4,6-diamidino-2-phenylindole (Merck) and sulforhodamine 101 (Sigma), and analyzed in a Partec PAS-2 cytofluorometer.

Transient Transfections—For transient transfections, the wild-type hamster TK promoter or a Smal–NheI fragment (564 base pairs) of the 5′-upstream sequence of the mouse TK gene (5) was cloned into the KpnI and NheI sites of the plasmid pGL3-Basic (Promega). The E2F-1 expression vector, pcCMV-HA-E2F-1(wt), was a gift of Dr. Wilhelm Krek; the DP-1 expression vector, pCMV-HA-DP-1, was kindly provided by Dr. Kristian Helin.

SAOS-2 cells (9 × 10⁴) or BHK cells (6.5 × 10⁴) were seeded in 3-cm diameter Petri dishes and transfected the following day with a total of 1.8 or 1.2 μg of DNA, respectively, using polyethyleneimine-assisted gene transfer (15). 6 μl of polyethyleneimine (10 mg of polyethyleneimine, M₉, 25,000) were diluted with 250 μl of 20 mM HEPES (pH 7.4) and added dropwise to 6 μg of DNA diluted in 250 μl of 20 mM HEPES (pH 7.4) with gentle agitation after the addition of each drop. After a 20-min incubation at room temperature, the appropriate amounts of DNA and polyethyleneimine complexes were added to the cells, which, prior to the transfection, had their growth medium replaced by 800 μl of serum-free medium. The transfection medium was replaced by fresh medium after 4 h. After 48 h, luciferase/β-galactosidase assays were performed using the Dual-Light system (Tropix Inc.) as recommended by the vendor.

Preparation of Nuclear Extracts—Crude nuclear extracts were prepared from exponentially growing BHK cells as described previously (16). Briefly, cells were washed twice with phosphate-buffered saline, collected in a conical tube, and centrifuged for 10 min at 1000 rpm. The cell pellet was washed once with ice-cold phosphate-buffered saline and resuspended in 3 volumes of hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). After 20 min on ice, the cells were homogenized using a syringe with a 27-gauge needle, and the nuclei were pelleted by centrifugation for 8 min at 5000 rpm. Nuclei were resuspended in 1 volume of lysis buffer (20 mM HEPES (pH 7.9), 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), and the lysate was incubated at 4 °C for 30 min with gentle mixing. The nuclear extract was obtained by centrifugation at 14,000 rpm for 30 min.

Preparation of Whole Cell Extracts—For the preparation of whole cell extracts, cells were washed twice with cold phosphate-buffered saline, collected in an Eppendorf tube, and centrifuged for 5 s. The cell pellet was suspended in 3 volumes of extraction buffer (20 mM HEPES (pH 7.9), 0.4 mM NaCl, 25% glycerol, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride were lysed by the freeze/thaw cycle in liquid nitrogen at −80 °C, respectively. After 20 min on ice, the extracts were obtained by centrifugation for 10 min at 14,000 rpm.

Electrophoretic Mobility Shift Assays—Binding reactions were carried out in a volume of 20 μl by incubating 10 μg of protein extract and 1 μg of salmon sperm DNA in a buffer containing 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM MgCl₂, 4% Ficoll, 0.1 mM EDTA, 0.1% Nonidet P-40, and 1 mM dithiothreitol. After 10 min on ice, end-labeled oligonucleotides were added, and the incubation was continued at room temperature for 20 min. This was followed by electrophoresis on a 4% polyacrylamide gel and autoradiography of the dried gel. For competition experiments, a 100-fold excess of unlabeled competitor DNA was incubated with the protein extract for 10 min before the labeled oligonucleotide was added. The sequences of the top strands of the individual oligonucleotide probes were as follows: E2F-like motif, 5′-CTCCGGTCTCTTGCGAGCAAGGAGGCCGCG-3′; GC box 1, 5′-CACAGGAGGAGGGGGCCCGGCACCACGCCC-3′; GC box 2, 5′-CACAGGAGGAGGGGGCCCGGCACCACGCCC-3′; GC box 3, 5′-CCCTGGCCACCGCCTGCTGCT-3′; and CCAAT box, 5′-GATCCGGTCTGGATTGCT-3′.

DNase I Protection Assays—The assays were performed as described (17) with the following modifications. The coding strand of the TK promoter fragment between positions −319 and +51 was end-labeled by filling in the NheI site at position +51. Binding reactions were carried out by incubating 30 μg of nuclear protein in a final volume of 50 μl of buffer containing 20 mM HEPES (pH 7.9, 60 mM KCl, 8% glycerol, 0.8 mM MgCl₂, 1 mM dithiothreitol, and 1 μg of poly(dI-dC). After 10 min on ice, the end-labeled DNA fragment was added, and the reactions were incubated for 20 min at room temperature before 3 units of DNase I were added, and digestion was allowed for 60 s. The DNase was inactivated by the addition of 0.6 mM NaCl, 0.2% SDS, and 10 mM EDTA, followed by phenol extraction and ethanol precipitation. The DNA was then analyzed on denaturing 6% polyacrylamide gels.

RESULTS

Isolation of the Hamster TK Promoter—The TK gene, the cDNA sequence, and the 5′-upstream region were previously identified from Chinese hamster cells (10–12). However, a functional analysis of the promoter sequence has not been carried out. The presumptive promoter sequence differs significantly from that of other rodents as well as from the human TK gene. In fact, there is little homology among six TK promoters, five mammalian and the chicken promoter (reviewed in Ref. 2). This contrasts with the high homology of sequences within both the transcribed regions of the genes and the more distant upstream regions. We used these homologous sequences to design oligonucleotides for polymerase chain reaction to isolate the TK promoter from BHK cells (derived from the Syrian hamster). As shown in Fig. 1, the promoter carries several GC motifs (potential binding sites for the transcription factor Sp1), one reversed CCAAT box, and a TATA element. In this respect, the sequence reported here is similar to the previously described 5′-upstream sequence of the Chinese hamster gene (11). Furthermore, a sequence with some resemblance to a binding site for E2F was discernible. This is of particular interest, since E2F was shown to play an important role in the regulation of the murine TK promoter (5–8) and may also be involved in the control of human TK (18, 19). We used the TK promoter from BHK cells to clarify which transcription factors are involved in the growth-dependent regulation of the hamster TK gene.

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FIG. 2. Identification of in vitro protein-binding sites in the hamster TK promoter by DNase I protection assays. The coding strand of the −319/+51 TK promoter fragment was 5′-end-labeled and used in the absence (−) or presence (+) of nuclear extract (Nucl. ext.) from exponentially growing BHK cells. A, footprint analysis of wild-type (wt) or mutated (m) promoter fragments containing different mutations, as indicated; B, competition experiment with Sp1 oligonucleotide. A 100-fold excess of an oligonucleotide containing the mouse TK promoter Sp1 site was added prior to the addition of the end-labeled wild-type promoter fragment. DNase I-protected sequences are indicated by brackets.

Positions −99 to −24—To identify protein-binding sites within the hamster TK promoter, we carried out DNase I protection assays. A clear protection of the three GC boxes and of the reversed CCAAT box was observed when the coding strand of the promoter fragment from positions −319 to +51 (relative to the transcription start site) was incubated with nuclear extract from exponentially growing BHK cells (Fig. 2A). To further map the region of protection contributed by individual transcription factors, single mutations were introduced in the binding motifs. The results shown in Fig. 2A indicate the binding of four protein complexes, which correspond to the number of putative binding sites, and that protein binding was abolished within the mutated element, whereas protection of other elements was not affected.

Sp1 Binds to the GC Boxes, and NF-Y Binds to the Reversed CCAAT Box—We next wished to define the transcription factor-binding sites within the upstream protected region. The region between positions −99 and −57 accommodates three GC boxes, representing potential binding sites for the Sp1 transcription factor family. Electrophoretic mobility shift experiments were performed with nuclear extracts from logarithmically growing BHK cells. Each of the GC-containing oligonucleotide probes produced three shifted complexes, which could be supershifted with antibodies against Sp1 and Sp3 (Fig. 3, B–D). All three complexes were competed by a 100-fold excess of an oligonucleotide containing the mouse TK promoter Sp1 site (Fig. 3E). The same result was verified in DNase I footprint experiments using the murine Sp1 probe in competition with the end-labeled wild-type promoter fragment (Fig. 2B). The fact that protein binding to GC boxes of the hamster TK promoter is strongly inhibited by an oligonucleotide carrying an Sp1/Sp3-binding motif present in another promoter, together with the supershift induced by Sp1- and Sp3-specific antibodies, provides compelling evidence for the conclusion that the region between positions −99 and −57 is indeed protected by three protein complexes of the Sp1 transcription factor family. Furthermore, it was demonstrated that the transcription factor binding to the CCAAT box is NF-Y, as antibodies against NF-YA and NF-YB, two of the three subunits of NF-Y, shifted the protein complex formed on the CCAAT oligonucleotide (Fig. 3A). An independent strong indication for an involvement of NF-Y in hamster TK promoter activity was obtained as follows. Promoter-luciferase reporter constructs were transfected into BHK cells alone or together with an expression plasmid carrying the information for a dominant-negative NF-YA subunit (20). This mutated NF-YA interferes with the formation of a functional, heterotrimeric DNA-binding complex. As shown in Fig. 4, the activity of the hamster TK promoter was significantly reduced by cotransfection of the dominant-negative NF-YA mutant, but not by cotransfection of wild-type NF-YA.

Interestingly, no protein complexes were detected with the E2F-like sequence within the hamster TK promoter, in contrast to what was found with the functional E2F-binding site from the murine TK promoter (Fig. 3E). More important, the binding activity for the hamster E2F-like sequence was absent in whole cell extracts from both quiescent and serum-stimulated BHK cells.

A Minimal Promoter of 122 Base Pairs Is Sufficient for Growth-regulated Expression—To examine which of the transcription factor-binding sites are essential for promoter activity and growth-dependent regulation, two approaches were taken. Luciferase reporter constructs were created, first with short-
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Prominent among these are the enzymes involved in DNA replication. The expression of a subset of genes is regulated during this transition. Our results indicate that the hamster TK gene is among these genes.

Promoter Strength and Growth-regulated Expression—

The TK promoter fragment (pGL3mTK) or the murine TK promoter (pGL3mTK) were transiently transfected into SAOS-2 osteosarcoma cells together with an expression vector for E2F-1. As expected, overexpression of E2F-1 transactivated the murine TK promoter, which was further stimulated by cotransfection of DP-1 or inhibited by pRb (Fig. 7, bars 5–10). In contrast, the activity of the hamster TK promoter remained unchanged when cotransfected with E2F-1 alone or together with DP-1 (Fig. 7, bars 1–3). These results strongly suggest that the hamster TK promoter is regulated in an E2F-independent manner.

**DISCUSSION**

Initiation of DNA replication requires the expression of a large number of genes at the G1/S boundary of the cell cycle. Prominent among these are the enzymes involved in DNA replication. The expression of a subset of genes is regulated during this transition. Our results indicate that the hamster TK gene is among these genes.

**Functional Requirement of cis-Acting Elements for TK Promoter Strength and Growth-regulated Expression—**

Having shown that GC box 3, the CCAAT motif, and the TATA box are sufficient for regulated expression of the promoter-luciferase constructs, these motifs were individually mutated, as indicated in Fig. 6C, to examine their involvement in promoter activity. Furthermore, because of the importance of a potential role of the upstream E2F-like sequence, we also mutated this sequence. Evidence that the mutations of the GC box and the CCAAT motif abolished protein binding is demonstrated by the DNase I protection assay (Fig. 2A). For all the mutations analyzed, this was also confirmed by mobility shift experiments (data not shown). The respective promoter-luciferase constructs were stably integrated into BHK cells, and the activity and regulation were analyzed using a pool of selected clones. In agreement with the results obtained with promoter constructs in which the E2F-like motif was deleted (Fig. 5), point mutations in this motif had little effect on promoter activity and growth-dependent regulation (Fig. 6, A and B). Together with the observation that no protein complexes were observed in electrophoretic mobility shift experiments with the E2F-like sequence (Fig. 3E), we can exclude that this upstream E2F-like element has a functional role in the expression of the hamster TK gene. In contrast, a dramatic reduction in hamster TK promoter activity was observed when either the TATA or CCAAT box was mutated (Fig. 6, A and B). Mutation of GC box 3 also led to a significant reduction in promoter activity (−16% of that of the wild type); however, this residual activity was still growth-dependent (Fig. 6B). This contrasts with the results obtained with the promoter construct carrying a mutated CCAAT box. This not only exhibited strongly reduced activity, but this activity increased maximally 2-fold upon serum stimulation. The transgene under the promoter with the mutated TATA element displayed background activity indistinguishable from that measured with an empty plasmid lacking any promoter. As expected for a promoter containing a TATA element, this is absolutely essential for promoter activity. Under these conditions, it is impossible to exclude any role of this element in growth regulation. However, if we mutated the TATA box within the context of the regulated minimal promoter such that it resembled the TATA element active in the growth-independent promoter of the muscle actin gene, this had no effect on promoter regulation (data not shown). Taken together, the results summarized in Figs. 5 and 6 show that GC box 3, the CCAAT box, and the TATA element are essential and sufficient to confer efficient promoter activity. They furthermore indicate that binding of NF-Y to the reverse CCAAT box is not only required for transcriptional activation, but also plays an important role in growth-dependent regulation of the hamster TK gene.

**In Contrast to the Murine TK Promoter, the Hamster TK Promoter Is Not Transactivated by E2F-1—**

Since it has been shown that members of the E2F transcription factor family, which heterodimerize with DP-1 and DP-2, are involved in the regulation of numerous genes that are expressed at the G1 to S phase transition (21), we investigated the possibility that E2F transactivates the hamster TK promoter through an as yet unidentified element. To test this possibility, reporter constructs containing the wild-type −319/+51 hamster TK promoter fragment (pGL3hTK) or the murine TK promoter (pGL3mTK) were transiently transfected into SAOS-2 osteosarcoma cells together with an expression vector for E2F-1. As expected, overexpression of E2F-1 transactivated the murine promoter, which was further stimulated by cotransfection of DP-1 or inhibited by pRb (Fig. 7, bars 5–10). In contrast, the activity of the hamster promoter remained unchanged when cotransfected with E2F-1 alone or together with DP-1 (Fig. 7, bars 1–3). These results strongly suggest that the hamster TK promoter is regulated in an E2F-independent manner.

**RESULTS**

**Fig. 4. TK promoter activity is repressed by a dominant-negative mutant of NF-YA.** BHK cells growing on 3-cm Petri dishes were transiently transfected with 1.2 μg of DNA from a mixture made up of 1 μg of wild-type hamster TK promoter-luciferase construct (pGL3hTK), the indicated amounts of expression vectors encoding dominant-negative NF-YA (pNF-YA29; Ref. 20) or wild-type NF-YA (pGL3mTK), or the indicated amounts of expression vectors encoding dominant-negative NF-YA (pNF-YA29; Ref. 20) or wild-type NF-YA (pGL3mTK). 0.5 μg of pCMVβgal as an internal control for transfection efficiency, and pcDNA3 empty plasmid to bring the total amount of the DNA mixture to 3 μg. The relative activity of luciferase to β-galactosidase is presented. The means ± S.D. of two independent experiments are shown as -fold induction of the activity obtained with the TK promoter-luciferase construct transfected alone.

**Fig. 5.** TK promoter activity is regulated by Sp1 and NF-Y. This indicates that one of the mutations in this motif had little effect on promoter activity and growth-dependent regulation (Fig. 6, A and B). Together with the observation that no protein complexes were observed in electrophoretic mobility shift experiments with the E2F-like sequence (Fig. 3E), we can exclude that this upstream E2F-like element has a functional role in the expression of the hamster TK gene. In contrast, a dramatic reduction in hamster TK promoter activity was observed when either the TATA or CCAAT box was mutated (Fig. 6, A and B). Mutation of GC box 3 also led to a significant reduction in promoter activity (−16% of that of the wild type); however, this residual activity was still growth-dependent (Fig. 6B). This contrasts with the results obtained with the promoter construct carrying a mutated CCAAT box. This not only exhibited strongly reduced activity, but this activity increased maximally 2-fold upon serum stimulation. The transgene under the promoter with the mutated TATA element displayed background activity indistinguishable from that measured with an empty plasmid lacking any promoter. As expected for a promoter containing a TATA element, this is absolutely essential for promoter activity. Under these conditions, it is impossible to exclude any role of this element in growth regulation. However, if we mutated the TATA box within the context of the regulated minimal promoter such that it resembled the TATA element active in the growth-independent promoter of the muscle actin gene, this had no effect on promoter regulation (data not shown). Taken together, the results summarized in Figs. 5 and 6 show that GC box 3, the CCAAT box, and the TATA element are essential and sufficient to confer efficient promoter activity. They furthermore indicate that binding of NF-Y to the reverse CCAAT box is not only required for transcriptional activation, but also plays an important role in growth-dependent regulation of the hamster TK gene.

**Fig. 6.** Effects of mutations in cis-acting elements on TK promoter activity. Bars 1–3 show the activity of the CK promoter such that it resembled the TATA element active in the growth-independent promoter of the muscle actin gene, this had no effect on promoter regulation (data not shown). Taken together, the results summarized in Figs. 5 and 6 show that GC box 3, the CCAAT box, and the TATA element are essential and sufficient to confer efficient promoter activity. They furthermore indicate that binding of NF-Y to the reverse CCAAT box is not only required for transcriptional activation, but also plays an important role in growth-dependent regulation of the hamster TK gene.
replication and precursor production as well as G₁ and S phase-specific cyclins, especially cyclins E and A. In many cases, a family of transcription factors, called E2F, is found to be involved in this regulation. E2F binds as a heterodimer with another group of proteins, called DP, to a DNA-binding motif present in the promoters of many of the genes expressed at the transition from G₁ to S phase and is regulated by the retinoblastoma protein pRb or its relatives p107 and p130 (21). One of the more intensively studied examples of a gene regulated during the cell cycle is the TK gene. Previous studies of the 5’-upstream region of the murine TK gene have supported a central role of the E2F family members in this regulation (5–8). Although the human TK promoter contains an element that resembles an E2F-binding site, the transcription factor binding to this site was reported to be distinct from the known E2F proteins (22). In fact, a clear involvement of E2F has so far only been proven for the regulation of the murine TK gene. Furthermore, a comparison of the promoter sequences of six vertebrates revealed astonishing differences (2), suggesting that each promoter is regulated by a distinct mechanism.

In this report, we describe the first detailed functional analysis of the hamster TK promoter. The promoter region was isolated from BHK cells. Analysis of the sequence revealed similarities to the human TK promoter in that both contain a TATA box and a CCAAT box, several GC motifs, and an element resembling an E2F-binding site. This structure is different from that of the murine TK promoter, which lacks both a TATA box and CCAAT motifs. However, in contrast to what has been shown for the human promoter (18, 19), the deletion and mutational analyses presented here clearly show that the E2F-like binding element does not play any functional role in the activation or regulation of the hamster TK promoter (Figs. 5 and 6). More important, overexpression of E2F-1 could not transactivate the hamster promoter, as could be demonstrated with the murine TK promoter (Fig. 7). Taken together, these results strongly suggest that the hamster TK promoter is regulated by a mechanism distinct from both the human and murine ones and independent of E2F.

We identified a 122-base pair fragment between positions –73 and +51 (relative to the transcription initiation site) as a minimal promoter, which conferred both efficient and serum-responsive expression of a reporter gene (Fig. 5). Within this region, a single binding site for Sp1, the CCAAT box, and the TATA box are present. Furthermore, it was apparent from mutational analysis that all three elements contribute significantly to the promoter strength and that the CCAAT box is important for promoter regulation (Figs. 5 and 6). Electrophoretic mobility shift assays identified NF-Y as the transcription factor that binds to the CCAAT box (Fig. 3A). Evidence that this transcription factor is functional at the hamster TK promoter was obtained with the help of a dominant-negative mutant that inhibited expression of a promoter-transgene construct (Fig. 4).

NF-Y, also named CBF or CP1, is composed of three subunits, A, B, and C, all of which are required for DNA binding (reviewed in Ref. 23). The sequence of the subunits is highly conserved (with >90% identity) among different mammalian species. NF-Y was initially identified as a transcription factor that interacts with the CCAAT motif of the major histocompatibility complex class II promoter (24). Interestingly, NF-Y was

![Deletion analysis of the TK promoter.](image)
found to recognize CCAAT boxes in several growth-regulated genes, including murine ribonucleotide reductase R2 (25), murine E2F-1 (26), cyclin B1 (27), cdc2, cyclin A, the protein phosphatase cdc25C (28), and human TK (29–31). The S- and G2-specific cyclin A, cdc2, and cdc25C genes also contain a downstream CDE/CHR element that consists of a very conserved sequence, \[(G/C)GCGGN5TTGAA\], which has been shown to be occupied only in G0 and G1 and to act as a transcriptional repressor of the transactivating activity of upstream binding sites, including the NF-Y recognition site (28). Since this element is absent in the promoter sequence described here, it is highly unlikely that this is the mechanism through which the hamster TK promoter is regulated.

The human TK promoter carries two CCAAT elements, to which NF-Y binds and which were shown to be important for both activation and regulation of the promoter (29–31). One report has suggested that the CCAAT displacement protein CDP/cut plays a role in this regulation (31). CDP/cut binds to sequences encompassing two CCAAT elements and acts as a repressor by preventing the interaction of the CCAAT-binding factor (17). It was shown that CDP/cut binds through the two CCAAT boxes in the human promoter when overexpressed in quiescent cells, thereby replacing bound NF-Y and exerting negative regulation (31). Interestingly, in vivo footprinting experiments on the human TK promoter showed that the E2F site, the GC boxes, and both CCAAT motifs are protected throughout the cell cycle (32), indicating that proteins are bound to these sites at all times, a situation reminiscent of that at the mouse TK promoter (8). The fact that the hamster TK promoter contains only one CCAAT box renders it unlikely that CDP/cut plays a role in this case. Moreover, we were not able to detect binding of CDP/cut to the promoter sequence reported here in mobility shift assays (data not shown). This adds additional support to the idea that the hamster and human TK promoters are regulated differently.

The hamster TK promoter seems to be regulated by a single Sp1-binding site and one CCAAT box, which leads one to speculate that, in this promoter, NF-Y replaces the function of E2F,
which has been demonstrated to cooperatively regulate the murine TK promoter with Sp1. Cooperative binding of NF-Y and Sp1 has been described in the regulation of other promoters (33, 34). Although Sp1 was not required for growth-dependent regulation of the hamster TK promoter (Fig. 6B), this element plays an important role in promoter strength (Fig. 6A). It is therefore possible that Sp1 cooperates with NF-Y to enhance the growth-regulated expression of the hamster promoter. Since NF-Y also recognizes CCAAT elements present in non-cell cycle-regulated promoters, growth- and cell cycle-specific regulation through NF-Y must certainly be dependent upon its promoter context. Therefore, the proximity of the NF-Y-binding site in the hamster TK promoter to the Sp1 and TATA elements might be very important in this particular setting. There is evidence for an important role of NF-Y in establishing transcription complexes and for recruitment of upstream DNA-binding factors (35, 36). In light of the observations that E2F (37), pRb (38–40), and Sp1 (43) interact with histone-modifying enzymes, it is interesting that NF-Y was shown to associate with the histone acetyltransferases P/CAF (41) and p300/CBP (42). It is therefore possible that the common strategy for regulating the murine and hamster and possibly other TK promoters involves growth-dependent changes in chromatin structure through local histone modification.

Acknowledgments—We thank Diane Mathis for providing antibodies against NF-YA and NF-YB; R. Mantovani for the expression plasmids NF-YA29 and NF-YA13; and Hans Rotheneder, Egon Ogris, and Christian Seiser for discussions.

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