Presence of regulatory sequences within intron 2 of the mouse thymidine kinase gene

Hans Rotheneder, Martin Grabner and Erhard Wintersberger*

Institut für Molekularbiologie, Universität Wien, Wasagasse 9, A-1090 Wien, Austria

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ABSTRACT

The intron 2 of the murine thymidine kinase (TK) gene was observed to contain two DNase hypersensitive site. *In vitro* footprinting experiments indicated specific binding sites for nuclear proteins which were characterized within the sequence of intron 2. Two GC boxes (binding sites for transcription factor SP1) and two new protein binding regions, one at the promoter proximal end of intron 2, the other one close to the border to exon 3 were found. Oligonucleotides were synthesized comprising the two new binding sites and were shown in gel mobility shift experiments to be capable of forming specific complexes with nuclear proteins. These proteins are present in growing as well as in quiescent cells suggesting that the sites described here do not contribute to growth regulation of TK expression. That they might play a role in upregulation of TK expression is, however, indicated by the results of CAT assays in which inclusion of downstream sequences of the TK gene containing parts or all of intron 2 were found to positively modulate the activity of the TK promoter.

INTRODUCTION

When cultured cells enter S phase during the cell cycle (G1/S transition) or after stimulation of arrested cells by growth factors (Go/S transition) many enzymes involved in DNA synthesis are induced. One of the more extensively studied enzymes of this group is cytoplasmic thymidine kinase (TK) whose activity increases up to 30 fold at the beginning and during S phase (1–4). Recent studies of a number of groups indicate that the regulation of TK takes place at several levels (5–19). Initiation of transcription being important largely during the Go to S transition of quiescent cells which were growth-stimulated by addition of serum whereas post-transcriptional regulation appears to predominate in cycling cells. Upstream sequences exhibiting growth dependent promoter activity were partially characterized in the human (20–22), hamster (23) and mouse (12, 15, 18, 24) TK gene. Contrary to the human and the hamster TK promoter the upstream region of the murine TK gene lacks a CCAAT box as well as a TATA element; all three promoters share the presence of GC boxes, binding sites for transcription factor SP1.

We have previously determined the sequence of about 500 bp upstream of the initiation codon of the murine TK gene (15) and, have observed that this sequence carries aside of the TK promoter another one active in opposite direction (24), controlling a hitherto unknown gene which, contrary to TK, seems not to be expressed in fibroblasts. The two divergently active promoters appear to exhibit little overlap. In order to further characterize the 5' end of the TK gene we have now determined the DNase sensitivity of chromatin at the 5' end of the TK gene and found that there are three DNase hypersensitive sites in the TK gene, one of which is localized in the promoter region while the other two are situated within intron 2. *In vitro* footprints have confirmed these results and allowed to define protein binding sites within the promoter and in intron 2 which were functionally characterized using the chloramphenicol acetyltransferase (CAT) gene as reporter. None of the regulatory regions within TK intron 2 exhibit growth dependence.

MATERIALS AND METHODS

Cells

Mouse 3T3 or 3T6 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics and 10% fetal calf serum. Cells were arrested by reducing the serum concentration in semiconfluent cultures to 0.2% and keeping them under these conditions for 3 days.

Cloning techniques

The methods used for the construction of DNA clones were standard (25). TK cDNA and genomic clones used in this work were described earlier. For the production of CAT constructs the *gpt* gene from pMSG-CAT (Pharmacia) was removed and replaced by the *neo* gene (originating from pSV2-neo, ref.26) and the MMTV promoter was removed and replaced by (i) the *EcoRI* to *Asp700* promoter fragment (ii) the *EcoRI* to *BamHI* fragment or (iii) the *EcoRI* to *NheI* fragment (see the schematic drawing in Fig. 1). DNA sequences were determined by the dideoxy method (27).

Determination of nuclease sensitivity

Nuclei were isolated from logarithmically growing or quiescent, serum starved 3T3 cells and incubated with various concentrations

* To whom correspondence should be addressed

of DNaseI as described (28). DNA was then extracted, cleaved with *EcoRI* and fragments separated by electrophoresis in 1% agarose gels. After transfer to Nylon membranes, blots were hybridized with labelled probes from the 5' part of the TK gene and further upstream sequences. The hybridization solution contained 5×Denhardt's solution, 1.5% sodium dodecylsulfate, 10 mM EDTA, 50 mM phosphate buffer (pH 8.0), 5×SSC, 50% formamide, 200 µg/ml of salmon sperm DNA and the labelled probe (about 50×10⁶ cpm). Hybridization was done over night at 43°C. After autoradiography, filters were treated with 50% formamide in 10 mM phosphate buffer (pH 6.8) for 60 min at 70°C prior to hybridization with a different probe.

Preparation of nuclear extracts for DNase protection and mobility shift assays

3T3 cells were scrapped off the dishes, pelleted and suspended in buffer (40 µl/per cell equivalent from one 10 cm Petri dish) consisting of 10 mM Hepes (pH 8.0), 0.5 mM spermidine, 0.15 mM spermin, 0.1 mM EDTA, 0.25 mM EGTA, 5 mM NaCl, 2 mM dithiothreitol, 10% glycerol and 2.5 units/ml of aprotinin. They were disrupted by homogenization in a Dounce homogenizer and crude nuclei were collected by centrifugation. These were resuspended in the same buffer to which Triton X100 was added to 0.25% and purified by centrifugation through 30% sucrose in the same buffer. DNase protection (footprint) and mobility shift experiments were carried out exactly as described (29). Poly d(AT) (3 µg) as well as sonified salmon sperm DNA (1 µg) were added to the mobility shift assays to avoid unspecific binding of the labelled oligonucleotide.

Transfection of CAT constructs, selection of stably transfected cells and CAT assays

Logarithmically growing cultures of 3T6 cells (in 60 mm Petri dishes) were transfected with about 50 ng of the individual CAT

constructs using the polybrene-method as described (30). Stably transfected cells were selected by addition of G418 (500 µg/ml) and resistant clones from one Petri dish were pooled, expanded and used for CAT assay. Cells were washed on the dishes with cold PBS, collected with a rubber policeman, suspended in 50 µl of 0.25 M Tris (pH 7.5)/cell equivalent from one 10 cm dish and disrupted by 3 cycles of freezing and thawing. After 5 min incubation at 65°C, debris were removed by centrifugation. 30 µg of protein from these extracts were used for the CAT assays as described (31).

RESULTS

Functional analyses of the murine TK promoter have shown that regulation of growth dependent transcription is confined to about 150 bp upstream of the initiation codon (12, 24). We have furthermore noticed that a region of about 500 bp upstream of

Figure 1: DNase sensitive regions within the 5' part of the TK gene. At the left part there is a schematic representation of the relevant genomic region of the murine TK gene, including upstream sequences (at the bottom), exons 1 to 3 (hatched) approximate locations of nuclease sensitive sites (filled squares) as well as the major fragments seen with the various probes. The arrow depicts the approximate initiation site and the direction of transcription. The probes (a,b, and c) used for hybridization are also indicated. Autoradiogram: Nuclei from 10⁶ 3T3 cells were incubated for 5 min at room temperature with 1 unit of DNaseI. DNA was then isolated and digested to completion with *EcoRI*. Nuclei were isolated from growing cells except for the experiment in lane 2 where serum-starved cells were used. Lane 5 shows the control band obtained without DNase digestion. Lanes 1, 2 and 5 were hybridized with probe c (the *EcoRI* to *NheI* fragment), lane 3 with probe b (the *EcoRI* to *PstI* fragment), lane 4 with probe a (the *EcoRI* to *Asp700* fragment). The sizes of fragments indicated at the left were estimated using a *HindIII* digest of lambda DNA as markers (not shown).

Figure 2: DNase footprints. A: Top: Schematic drawing of the mouse TK genomic region up to intron 3. A, B and C are diagnostic footprints showing protected sequences. A: Protection of sequences within the promoter. Lane A/G shows the sequence bands, lane 1 shows bands of unprotected DNA, lanes 2 and 3 show regions within the DNA protected by incubation with 50 µg of protein from a nuclear extract isolated from growing (lane 2) or quiescent (lane 3) cells. The sequence from -80 to -100 carries the GC box, the second protected sequence (-30 to -50) comprises the AT rich region close to the transcription start sites. B: The promoter proximal regulatory region within intron 2. Lanes A/G and A/C show sequencing bands, lanes 1 and 2 show unprotected DNA, lanes 3 to 8 show protection by, respectively, 6, 10, 16, 20, 35 and 70 µg of protein from a nuclear extract of growing cells, lanes 9 and 10 show protection by 10 and 20 µg respectively of protein from a nuclear extract of quiescent cells. The protected region between nucleotide 250 and 275 is one of the novel regulatory sequences. The protected region around 350 is one of the GC boxes. C: The promoter distal regulatory region within intron 2. Lane A/G shows the sequence bands, lane 1 shows unprotected DNA, lane 2: incubation of the DNA with 70 µg of protein from a nuclear extract of growing cells, lanes 3 and 4 incubation with 70 µg of protein from nuclear extracts of quiescent cells. The sequence from nucleotide 908 to 945 is protected by high concentrations of nuclear protein. This region causes strong stimulation of transcription. There is a nuclease hypersensitive site at nucleotide 928.

the ATG for TK includes not only the TK promoter but in addition another promoter active in opposite direction. This promoter controls a so far unknown gene from which partial sequences were obtained and which seems not to be expressed in fibroblasts. In order to extend our analysis of the upstream region of the mouse TK gene we tested the DNase sensitivity of the chromatin using probes that span the TK promoter and the 5' region of the expressed part of the TK gene. Contrary to the promoter and parts of the coding region of the divergently oriented gene, where no DNase hypersensitive sites were detected (not shown), three such sites are present within the TK gene and its promoter (Fig. 1). One of these sites is located in the promoter around the multiple sites where transcription starts, two further sites were found in intron 2. No significant difference was apparent in the nuclease sensitivity pattern of growing or quiescent cells.

In order to correlate the DNase hypersensitive sites with possible binding sites for transcription regulators, *in vitro* footprint analyses were carried out. Using probes spanning the sequences

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-150  GGTGAGCTC CAGGCTTTC AGTACCTGA GAGTGGAC GATCTCTCT TCTCTCCGC CCTCTTTCG TTGCGGGCA AATGAGACA GTAGTGGAA
-50  ATTTTTCAC CCAGGACTC TGGTCTTAA CTAGGTTTG CACAGAGCC ATGAGCTACA TCAATCTGC CACCTCTCTG CCGAGTCCC CCGAGGAGC
    Bcl1/Inf1
51  TCGGGGCGAG ATTCAGCTGC GGGTCTCTGA GTGGGCTGG GTGGTGGCTG GTGCGGGTGT TACAGGGGG AGACCCCTTA AATGACTTCT CTCTAGGTT
    Bcl2/Inf2
151  ATTTCTGGGC CATGTTTCT AGGGAAGAG TAATGAATG GCTTCGGGG CAGTGGGCT CCTCTCTCT CTGAGCTCT TCTCTCTGAG GCGCTCTCT
251  ACTTATCTCC GATTCGGGAG GTTCTGAGA GCTCTGACT AGCGCGAAA CCGCTCGAG CTCTGTGTGT ACATTCTGGC GGAATTTTGG ATTCTCTGA
351  TTCTGTCAT CTCTCTGCC AAGCTTTTC TCTTGGAGC CCCTATCTT GCTGAGGGG ACCAGATGG GGTACATTTG AAGACCCGAG CCGACATTC
451  CCAGAGGCC ACCGAGCTT CATGTCTCC TGGAGTTAG GCACCTCTC TACAGTTTC CTGATTCAT CCGCATCTGA GCTCTCTTAA AGCTTTGGG
551  CCGATTCAC TCTGAGTGA GGTCTTAAC TCTGGAGTG CACCTCTCT TACAGAGCA ACATCTCTT GGTCTCTTCT GCTTATCTT GGGTTAGTT
651  TTTTCTTTT TTTTCTTCT CAGCTCTTT ATTCTGGCC CAGCTCTCT ATCCCTTGC CTCTCTCTG GTGATCTGC ATACAGGCA CTTATTTAT
751  TCTGATGTT CTGGTCTAC CTGATTTGT CCGTCTGCC GAGGTGAGG GCGAAGCTA TGAATCGGA TCGAGAGCA AGCTCTCTG TGAATCTCT
851  AAGACTTTG AAGTCTATC ATGAAATTT AATTCTTCT AGAGAGCAT GGGTGAAGC GCTGAGTTT CCGTCTGCC ACGTATGTT GTATCTGGG
    Inf2/Inf3
951  CCGGCTTTT GCTCGGGCT CATGTCTCT GTTATTTCT TCGA 955
  
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Figure 3: Nucleotide sequence of 150 bp upstream region, of exons 1 and 2 and introns 1 and 2 of the mouse TK gene. Exon-intron boundaries are indicated as well as the two GC boxes in intron 2 (underlined) and the two regions within intron 2 characterized by DNase hypersensitivity and footprinting analysis (doubly underlined). The sequence from nucleotide 1 to 995 which contains the hitherto unpublished sequence of introns 1 and 2 was submitted to the EMBL Data Library and has the accession no. X60980.

Figure 4: Gel shift analysis using oligonucleotides corresponding to the footprints within intron 2 (Fig. 2). Lanes 1 to 4 show the bandshift of the 26 bp long oligonucleotide containing the sequence from base 250 to 275, lanes 5 to 8 that of the 42 bp long oligonucleotide containing the sequence from base 909 to 950 (Fig. 3). About 1 ng of labelled double stranded oligonucleotide was incubated without protein (lanes 1 and 5) or with 5 μ g of protein from nuclear extracts of quiescent cells (lanes 2 and 6) or from logarithmically growing cells (lanes 3 and 7). A hundredfold molar excess of the respective unlabelled oligonucleotide was added in lanes 4 and 8 to prove specific binding.

of interest and nuclear extracts from growing or serum-starved, arrested cells, we found that within the promoter sequence there was strong protection of and around the GC box (nucleotides -80 to -100) suggesting that binding of SP1 and possibly of other transcription regulators recognizing sequences nearby the GC box is required for TK expression (Fig. 2). In addition there is protection of sequences near the AT rich region around nucleotide -50. This region may correspond to the nuclease sensitive site within the promoter and encompasses one major site of transcription initiation (our cDNA clone starts with nucleotide -56). The footprints were the same whether produced by nuclear extracts from resting or growing cells. Protection of both of these sites within the murine TK promoter was also reported recently by Dou et al.(18).

Interestingly, we also found defined areas of nuclease protection in *in vitro* footprints within intron 2. Aside of protection of the two GC boxes (see Fig.3 for the sequence), one of which is shown in Fig. 2 (part B, nucleotides 330 to 350), there are protected sequences at the beginning (nucleotides 250 to 275) and the end (nucleotides 908 to 950) of intron 2, approximately corresponding the DNase sensitive sites of chromatin (Figs. 2B and 2C show the diagnostic parts of the footprint). Again, the footprints were not significantly different if nuclear extracts were prepared from growing or quiescent cells. It should be mentioned, however, that the amounts of protein from the nuclear extract necessary to obtain protection was considerably different for the two regions. Whereas very small amounts of protein sufficed to see protection in the 5' part of intron 2 (Fig. 2B) much higher concentrations were necessary to protect the region close to exon 3 (Fig. 2C). On the other hand, this footprint very clearly shows the DNase hypersensitive site within this area of the gene (nucleotide 928).

Full characterization of these newly discovered binding sites within intron 2 required the determination of the nucleotide sequence of this part of the TK gene. Since, so far, only exon sequences of the gene are known, we determined the sequence of intron 1 and 2 and these are shown in Fig. 3, together with the sequences of exon 1 and 2 (the exon-intron boundaries are indicated) and 150 bp of the upstream region containing regulatory promoter sequences. The two GC boxes are

Figure 5: CAT assays. The CAT gene was under the control of the TK promoter E/A (*Eco*RI -555 to *Asp*700 -51), the fragment E/B ranging from the *Eco*RI site to the *Bam*HI site in intron 2 (803) which includes the two GC boxes and the promoter proximal protein binding site in intron 2 or the fragment E/N ranging from the *Eco*RI site to the *Nhe*I site (at the beginning of intron 3, see the schematic drawing in Fig. 2). Equal amounts of protein from total cell extracts were used and the two series of assays using growing cells were done with independently produced extracts.

underlined, the regions protected by hitherto unknown proteins and corresponding to the DNase hypersensitive sites are doubly underlined.

The next question was whether these sequences indeed bind proteins present in nuclear extracts. To answer this question, oligonucleotides were synthesized corresponding to the sequences doubly underlined in Fig. 3. These were used in gel shift analyses using nuclear extracts from arrested or growing fibroblasts. As shown in Fig. 4, both sequences form nucleoprotein complexes which in agreement with the footprint analysis, were independent of the growth condition of the cells. Again, formation of complexes occurred more readily with the 5' binding site than with the one close to exon 3. The specificity of complex formation was proven by the efficient competition by an excess of unlabelled oligonucleotide.

Preliminary results suggesting that intron 2 sequences might modulate the activity of the TK promoter were obtained in analyses using the CAT gene as a reporter under the control of the promoter only (*EcoRI* to *Asp700*) or of constructs including sequences of the expressed part of the TK gene, the fragment from *EcoRI* to *BamHI* (which contains the 5' binding site in intron 2 as well as both GC boxes, see Figs 2 and 3) or the fragment from *EcoRI* to *NheI* (which in addition includes the binding site at the 3' end of intron 2). The respective constructs were stably transfected into 3T6 cells (using the *neo* gene cloned into the CAT containing plasmid as selectable marker) and pools of G418 resistant cells were used for the assays. This not only allowed an analysis of the growth dependence of CAT expression more reliably than with a test employing transient transfection but also avoided differences due to varying transfection efficiencies. The use of pools of stably transfected cells should average differences possibly existing within individual clones. As can be seen in Fig. 5, CAT expression from the intrinsically weak murine TK promoter (12, 24) is significantly stimulated when intron 2 sequences are included in the control region. This is particularly evident for sequence close to exon 3. If cells were made quiescent by serum starvation, expression of CAT is low irrespective of the construct used. The relative expression efficiency, however, remains similar to that of growing cells. This is to be expected as it was shown previously that the promoter itself is responsible for growth dependent expression (12, 24) while all the data presented above suggest that the binding sites within intron 2 are occupied in a growth independent manner.

DISCUSSION

Examination of DNase sensitive sites in chromatin including the promoter region and the 5' end of the expressed part of the murine TK gene revealed the presence of such sites not only in the promoter but also within intron 2. Further analyses using DNase footprints and gel mobility shift assays complemented by sequence determination confirmed the presence of two SP1 binding sites and of two other protected sequences (one at the 5' end, the other one at the 3' end) in intron 2. Both of these newly characterized sequences function as binding sites for nuclear proteins. Addition of these intron sequences to CAT constructs led to a marked stimulation of expression over that seen with the promoter only.

Several examples for a modulation of the promoter activity in a positive or a negative way by sequences within the expressed part of a gene have recently been described (32–39). In some cases positive modulation like that described here was observed involving genes which are under the control of a promoter lacking

a TATA element; a most relevant example being the gene coding for dihydrofolate reductase (DHFR, refs. 40, 41). Not only is this gene regulated with growth very much like the TK gene but, in addition, Farnham and Means (40) identified the sequence CCCCCTGCCATC as a positive downstream regulator of DHFR activity in the murine DHFR gene. Sequences homologous to this one were found not only in other DHFR genes (human and hamster) but also downstream of several other promoters. Interestingly within the region of intron 2 of the murine TK gene (around nucleotides 250–260) which gives rise to a strong footprint and a DNase hypersensitive site (see Figs. 1 to 3) there is the related sequence CCCCTCCCCATC. Intron sequences (with the exception of exon-intron boundaries) are not published as yet for the human or the hamster TK gene. It is therefore unknown whether they contain a similar motif. Other examples of growth regulated genes whose expression is modulated by intron sequences are thymidylate synthase (42) and proliferating cell nuclear antigen (43). It is not yet known which sequence elements in these introns are responsible for their activity. On the other hand, it was reported, that introns do not contribute to the regulation of the human (44), the chicken (45) and the hamster (6) TK gene. We are currently constructing a series of mouse TK minigenes, incorporating various introns, for a more precise study of the role of downstream sequences in the regulation of TK gene expression.

While this paper was in preparation, the work of Fridovich-Keil et al. (46) came to our attention in which a role for a sequence downstream of the translation initiation site (to nucleotide 159, which is in midst exon 2 and excludes the regions described by us) in the expression of the murine TK gene was reported. It is so far unknown which elements are responsible for this activity. Two of our CAT constructs (those in which the CAT gene is under the control of the *EcoRI* to *BamHI* or the *EcoRI* to *NheI* fragment, see Fig. 1) incorporate this region. As regards the increase in CAT expression when the *EcoRI* to *BamHI* fragment is used rather than the promoter only (*EcoRI* to *Asp700*) we can not so far differentiate between a possible role of sequences 5' to intron 2 (present in the construct in ref. 46) and sequences within the 5' half of intron 2 (described in this paper). However, it is quite clear from our CAT assays (Fig. 5) that sequences past the *BamHI* site in intron 2 stimulate expression further. Studies using defined minigenes and mutation analyses of protein binding motifs within intron 2 should clarify the mechanism of modulation of the activity of the murine TK promoter by downstream sequences.

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