Bidirectional promoter activity of the 5′ flanking region of the mouse thymidine kinase gene

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The 5′ flanking region of the gene coding for cytoplasmic thymidine kinase (TK) in the mouse (a total of 490 bp upstream of the initiation codon) was tested for promoter activity using the chloramphenicol acetyltransferase gene as reporter. It was found that the region can be divided into two parts, one of which carries promoter activity in the direction of TK, whereas the 5′-half has promoter activity in the opposite direction. A fragment of 140 bp was sufficient for growth-dependent promoter activity in the direction of TK, although about 100 bp further upstream, enhanced the activity. Expression from the divergent promoter was independent of cell growth.

Thymidine kinase; Mouse fibroblast; Promoter acitivity; CAT assay

1. INTRODUCTION

Thymidine kinase (TK) is an enzyme of the salvage pathway whose activity is regulated with growth and during the cell cycle at the transcriptional level as well as at several levels post-transcription [1-13]. We have previously isolated and characterized the mouse TK gene and pseudogene sequences from a cosmid library and determined the sequence of about 500 bp upstream of the initiation codon. This sequence shows little resemblance to other TK promoters, except for a stretch of 18 bp which is present (with only two modifications) in the upstream region of mouse, human and hamster TK genes [14]. There are, however, a number of sequences present in the mouse TK promoter which are known to be binding sites for transcription factors (outlined in Fig. 4 of [14]); in particular, there are two binding sites for transcription factor SP1. In order to learn more about the relative contribution of transcriptional and post-transcriptional processes during growth regulation of TK expression in cultured mouse cells, we have constructed plasmids containing various parts of the TK upstream sequence in front of the gene for bacterial chloramphenicol acetyltransferase (CAT) to test for promoter activity. Surprisingly we found that this upstream region contains, in addition to a growth regulated promoter in the direction of the TK gene, a divergent promoter active in the opposite direction. In CAT constructs the divergent promoter did not exhibit growth regulation.

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Abbreviations: TK, thymidine kinase; CAT, chloramphenicol acetyltransferase

2. MATERIALS AND METHODS

2.1. *Cells*

Mouse 3T6 or LMTK$^-$ cells were grown in Dulbecco-modified Eagle's medium containing antibiotics and 10% fetal bovine serum. Cells were arrested by keeping them for 3 days in medium containing 0.2% serum.

2.2. *CAT constructs and CAT assays*

Starting points for the preparation of the CAT constructs shown in Fig. 1 were the plasmid pMSG-CAT (Pharmacia-LKB) and the 2.7 kb *Eco*RI fragment containing the 5′ end of the mouse TK gene [14]. pMSG-CAT carries an MMTV promoter in front of the CAT gene which was removed by digestion with *Sma*I and *Hin*dIII and either blunt end ligated to yield p0-CAT or replaced by either one of the following upstream fragments: the 5′ *Eco*RI to *Asp*700 fragment (−490 to −52; [14]) in either direction, the *Nci*I to *Asp*700 fragment (−254 to −52), the *Nci*I to *Eco*RI fragment (−254 to −490), the *Sac*I to *Asp*700 fragment (−145 to −52) or the *Sac*I to *Eco*RI fragment (−145 to −490), to yield the plasmids pE/A-CAT, pA/E-CAT, pN/A-CAT, pN/E-CAT, pS/A-CAT and pS/E-CAT (Fig. 1). The orientation of the inserts was verified by restriction analysis. For the production of cell lines transformed by integrated CAT plasmids, the *gpt* gene present in the CAT plasmids (originating from pMSG-CAT) was removed and replaced by the *neo* gene from plasmid pSV2-*neo* under control sequences from SV40 [15], to allow selection of transformed cells with Geneticin.

For CAT assays, LMTK$^-$ cells were transfected with 10 μg of plasmid DNA by the calcium phosphate method as detailed by Gorman [16]. We have chosen LMTK$^-$ cells because the same cells could also be used for transfection with various constructs containing TK cDNA or TK gene sequences followed by HAT selection for TK$^+$ clones. Extracts were prepared 48 h later and assays carried out essentially as described [16] except that only 0.125 μCi of [^{14}C]chloramphenicol (Amersham) were used per reaction mixture. Equal amounts of protein from the various extracts were added in parallel assays. After autoradiography, spots of acetylated chloramphenicol were scraped from the thin layer plate and counted to calculate percent conversion of chloramphenicol into the acetylated product. Experiments were repeated at least 3 times. Cell lines stably transformed with various CAT constructs were produced by transfection of plasmids containing the *neo* gene into LMTK$^-$ cells followed by selection in the presence of Geneticin (600 μg/ml).

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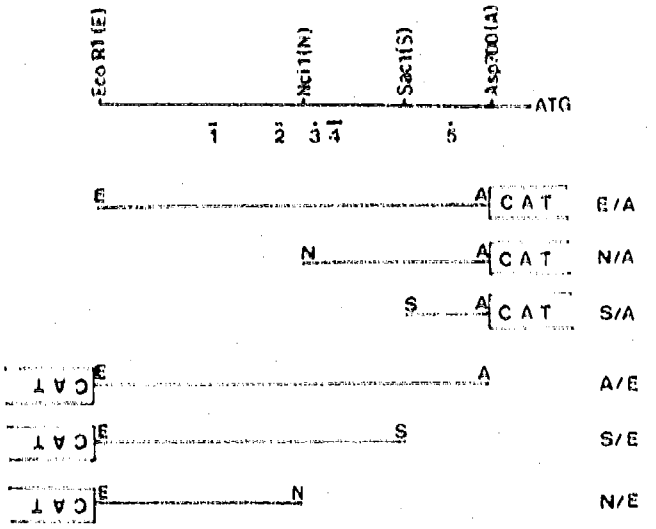

Fig. 1. Schematic representation of the upstream flanking region of the mouse TK gene (490 bp) and of CAT constructs produced. (Top) Location of restriction sites used; 1 to 5 are a selection of binding sites for transcription factors: (1) TATA box in the direction opposite to TK, (2) modified octamer box oriented in the direction opposite to TK, (3) and (5) SP1 binding sites (GC boxes), (4) the 18mer sequence conserved in the upstream region of mouse, hamster and human TK genes [14].

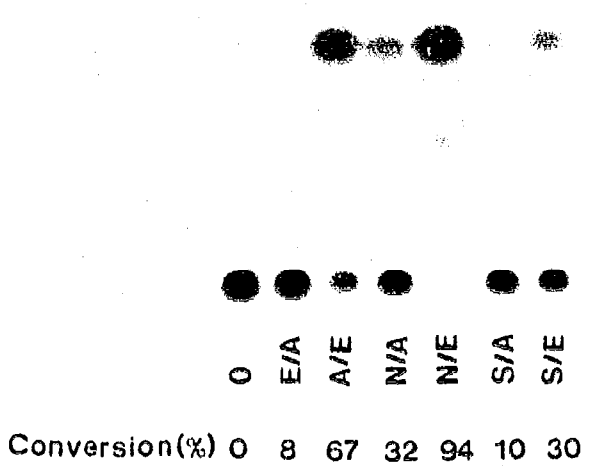

Fig. 2. Transient expression of CAT. Details of these assays are described in section 2. The constructs transfected into LMTK⁻ cells are defined in Fig. 1.

2.4. *Cloning of DNA sequences upstream of the TK gene*

The cosmid carrying the total TK gene (cosmid A in [14]) carries in addition to the TK gene more than 10 kb of mouse DNA upstream of the TK gene. This DNA was subcloned in the form of *Eco*RI, *Bam*HI and *Eco*RI-*Bam*HI fragments into plasmid pAT153 and the order of the fragments determined using standard procedures [17]. Partial sequence data were obtained by introducing fragments into M13 phages, using M13mp18 and M13mp19 (Pharmacia) as cloning vectors, followed by sequencing according to the dideoxy-method [18]. Southern and Northern transfers were carried out using standard procedures [17]. Probes to be used for hybridization of Southern or Northern blots were prepared by random primer labelling.

3. RESULTS AND DISCUSSION

The 490 bp TK upstream region (isolated from a mouse cosmid containing the TK gene) was tested for promoter activity in transient CAT assays, first using the fragment from the *Eco*RI (nucleotide −490 from the ATG) to the *Asp*700 site (nucleotide −52; our TKcDNA clone started at nucleotide −58, [3]; for the sequence of the 490 bp TK upstream region see [14]). If the fragment was inserted in front of the CAT gene in the TK orientation, it exhibited only low promoter activity, quite in contrast to the control, namely the same region inserted in the divergent orientation. Looking at the sequence it became apparent that the 5′ part carries sequence elements similar to a TATA bos and an octamer box oriented in the direction opposite to the TK gene. In order to test for the possible presence of two divergently oriented promoters in the region we constructed a series of CAT plasmids carrying parts of the sequence in either the direction of the TK gene or in the divergent orientation (Fig. 1). These were tested for CAT activity in parallel assays and the results are shown in Fig. 2. It is clear that the total fragment was more active in the opposite orientation (compare E/A with A/E); activity in the direction of TK increased upon removal of the 5′-half of the total sequence (leaving fragment N/A), while the removed sequence (N/E) had at the same time stronger activity than fragment A/E. Removing further sequences (carrying the conserved 18mer and one GC-box) in the direction of TK, and thus creating plasmid S/A, decreased the promoter activity in the TK direction and a similar decrease was observed when the remaining 5′ fragment S/E was tested for promoter activity in opposite direction. Hence, the sequences between the *Nci*I site and the *Sac*I site probably contain binding sites for transcription factors which increase the activity in the direction of the TK gene but are not absolutely required for TK promoter activity. The results suggest that the TK promoter and the promoter active in the opposite direction are not overlapping.

The next question addressed was whether the expression of CAT constructs in the direction of TK exhibited growth regulation in stably transformed cells. To this end the gene coding for Neomycin resistance was added to the CAT plasmids and LMTK⁻ cells transformed therewith were selected in the presence of Geneticin. As shown in Fig. 3, expression of CAT was growth-dependent in cells transformed by plasmids carrying promoter sequences in the direction of TK (N/A or S/A) whereas no such growth dependence was seen if expression was in the opposite direction (S/E in Fig. 3; an identical result was obtained with the N/E construct, not shown). As in transient assays, expression from the longer construct (N/A) was somewhat stronger than

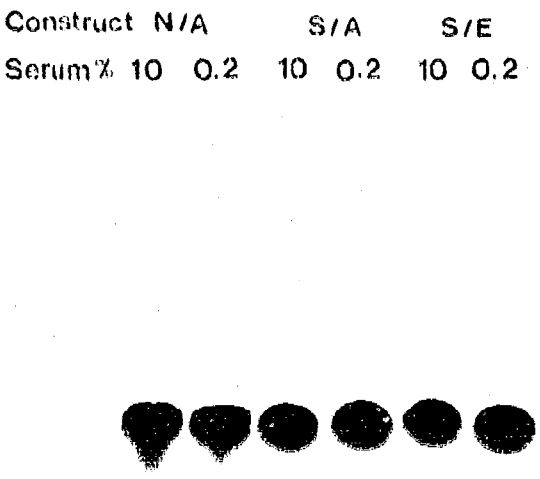

Fig. 3. Growth regulation of promoter activity. LMTK⁻ cells were transfected with CAT plasmids carrying the upstream region indicated (see Fig. 1) and the *neo* gene. Transformed cell lines were selected in medium containing Geneticin. Cells were arrested for 3 days in medium containing 0.2% serum. Half of the plates then received 10% serum for growth stimulation, and extracts were prepared 18 h thereafter.

that from the shorter one (S/A). Both exhibited dependence on growth, which indicated that sequences upstream of the *Sac*I site bind transcription factors which increase promoter activity in the direction of TK but may not be responsible for growth regulation of TK gene expression, a result which is in agreement with the report of Lieberman et al. [11].

Divergent promoter activity has recently been found in the upstream flanking region of several genes: dihydrofolate reductase [19–22], α(IV) collagen [23], c-Ha-*ras* [24], rat 3-hydroxy-3-methylglutaryl coenzyme A reductase [25], rat insulin II [26]. The most thoroughly studied example is that of dihydrofolate reductase in which case an overlapping divergently active promoter was shown to direct the expression of the dihydrofolate reductase gene and of a gene, *Rep-1*, coding for an enzyme possibly involved in DNA mismatch repair [27,28]. We herewith add another example of a bidirectional regulatory region, the mouse TK gene 5′ flanking sequence.

We subcloned some 10 kb of sequences upstream of the TK gene which were present on the cosmid carrying the TK gene. The restriction map of this region and the subcloned fragments are shown in Fig. 4A. Hybridization of these fragments to Southern blots of mouse genomic DNA revealed the presence of highly repetitive elements within the small *Xho*I to *Eco*RI fragment of UP1, within UP5 and UP9 and within the *Sma*I to *Sma*I fragment of UP6. These repetitive elements recognized each other, indicating that they were highly related. Indeed, sequencing of two of these (UP9 and the small *Xho*I to *Eco*I fragment of UP1) revealed almost identical sequence which showed 80% homology to the B1 elements, the mouse *Alu* equivalent (not shown). All

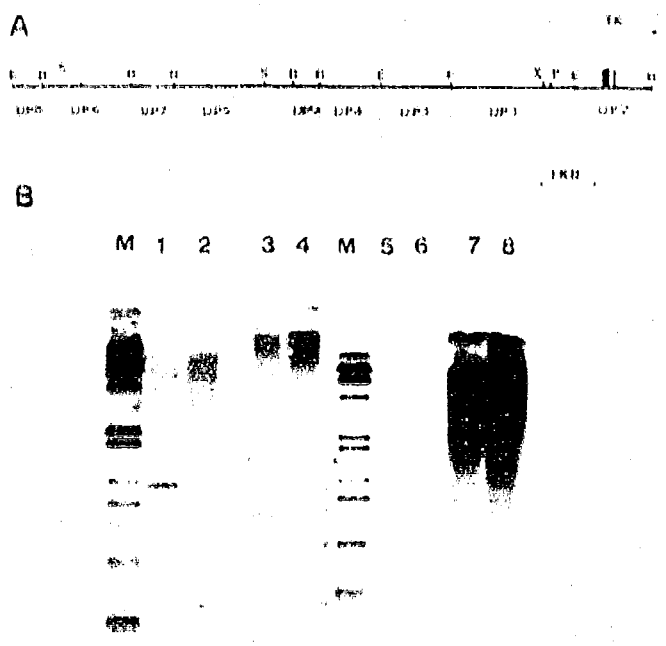

Fig. 4. Genomic region upstream of the TK gene. (A) Schematic drawing indicating representative restriction sites used for subcloning and the designation of clones. Abbreviations: B = *Bam*HI; E = *Eco*RI; P = *Pst*I; S = *Sma*I; X = *Xho*I. Vertical bars in the expressed region of the TK gene (within UP2) depict exons 1 and 2. (B) Examples of Southern blots of DNA from 3T6 cells (lanes 1, 3, 5 and 7) and from LMTK⁻ cells (lanes 2, 4, 6 and 8). (Lanes 1 and 2) *Eco*RI digests, probed with the UP3; (lanes 3 and 4) *Bam*HI digests probed with UP7; (lanes 5 and 6) *Eco*RI plus *Bam*HI digests probed with UP2; (lanes 7 and 8) *Eco*RI digests probed with the small *Xho*I to *Eco*RI fragment containing repetitive DNA sequences. M = marker (*Hin*dIII digest of λDNA plus *Hae*III digest of ΦX174 DNA).

the other fragments hybridized to single copy DNA (see Fig. 4B for examples). LMTK⁻ cells which lack the total TK gene [14] also lack the upstream sequences; they gave signals only with clones containing repetitive DNA (Fig. 4B). When the upstream subclones were hybridized to Northern blots of RNA isolated from mouse fibroblast cell lines, signals were obtained only with repetitive DNA (resulting in smears, not shown) but not when the probes contained only single-copy DNA. In order to look for signs characteristic of an expressible gene, we determined the sequence of part of fragment UP1 immediately following the presumptive divergent promoter. An open reading frame was found which codes for 45 amino acids and ends at a splice donor site. From this observation and from the presence of repetitive elements, we consider it likely that many of the sequences of the putative divergent gene which we have subcloned may represent introns. It may therefore be difficult to obtain conclusive signals in Northern analyses using these sequences, and further analysis will have to await the isolation of cDNA clones.

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