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The processed pseudogene of mouse thymidine kinase is active after transfection

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Aside of the gene coding for cytoplasmic thymidine kinase, the genome of mouse cells carries two pseudogenes. Both are inactive in situ. One of the pseudogenes is a processed pseudogene in which a two base pair deletion caused a shift of the reading frame and a shortening of the gene product from the 233 amino acids of thymidine kinase to 177 amino acids in the pseudogene product. We report here that introduction of this pseudogene into LTK$^-$ cells gave rise to cells with a thymidine kinase positive phenotype. The transformed cells carried multiple copies of the pseudogene the upstream region of which exhibited low but measurable promoter activity. Replacement of the upstream region of the pseudogene by a promoter of Simian virus 40 or of the mammary tumor virus resulted in high transfection efficiencies and in cell lines exhibiting high thymidine kinase activities.

Pseudogene; Thymidine kinase; Transfection

1. INTRODUCTION

The genome of many eukaryotic organisms contains aside of functional genes sequences which are related with a particular gene but exhibit a varying number of changes which result in inactivity. Such sequences were named *pseudogene* [1] (reviewed in [2,3]). Many pseudogenes lack introns and carry an oligo (dA)-oligo(dT) tract at the 3′ end followed by an about 15 base pair sequence that is repeated at the 5′ end. Such pseudogenes are termed *processed pseudogenes*; they probably arose by reverse transcription of a mRNA followed by integration of the DNA copy into the genome [2,3].

We found that the genome of mouse cells contains in addition to the functional gene for thymidine kinase (TK) two pseudogenes [4] one of which, a processed pseudogene, was sequenced [5]. The normal mouse *tk* gene codes for a protein with 233 amino acids. Compared to the sequence of the *tk* cDNA the processed pseudogene contains several single base changes, small deletions and insertions [3]. One deletion alters the reading frame after amino acid 177; thereafter follow 12 unrelated amino acids and then a stop codon. Only 3 changes in the pseudogene result in different amino acids within these 177 amino acids. The pseudogene is not expressed since LTK$^-$ cells, lacking the entire normal *tk* gene, do not exhibit any signal in Northern analysis [4] and do not revert to the TK$^+$ phenotype. We show here that the pseudogene, if transfected into LTK$^-$ cells, nonetheless can give rise to a TK$^+$ phenotype. Addition of either a Simian virus 40 (SV40) promoter or the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter greatly enhanced the frequency of appearance of TK$^+$ clones.

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2. MATERIALS AND METHODS

2.1. *Cell culture and transfection*

LTK$^-$ cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. One day prior to transfection LTK$^-$ cells were seeded at a density of 10^6 cells per 10 cm dish. Transfection was done by the calcium phosphate method [6] using 2 μg pseudogene-containing plasmid plus 10 μg of carrier DNA (from LTK$^-$ cells). Twenty-four hours after transfection the medium was changed to hypoxanthine-aminopterin-thymidine (HAT) medium [7] to select for TK$^+$ cells. Individual colonies were picked and expanded to mass cultures. For introduction of the Neomycin-resistance gene, cells were co-transfected with 2 μg of pseudogene plasmid DNA plus 10 μg of a plasmid carrying the bacterial Neomycine-resistance gene under the control of an SV40 promoter. Selection was in medium containing 600 μg/ml of Geneticine. Geneticine-resistant clones from one dish were combined and plated on HAT medium to select for TK$^+$ cells. Transfection with plasmids carrying the pseudogene under the SV40 or the MMTV promoter or transfection with a plasmid containing the HSV *tk* gene was carried out similarly. Selection was on HAT medium. For karyotype analysis, cells were blocked in metaphase by treatment with Colcemid, trypsinized and the chromosomes spreaded as described [8]. Chromosome preparations were stained with Hoechst 33258.

2.2. *Plasmid construction*

The total pseudogene sequence (the *Eco*RI to *Bam*HI fragment from cosmid C in [4]) was cloned in pUC18 using standard procedures [9]. For the introduction of viral promoter sequences, the *Pvu*II to *Bam*HI fragment was blunt end ligated into: (1) the *Sma*I site of

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plasmid pSVL (Pharmacia) which carries the SV40 late promoter; or (2) the large *Sma*I to *Hpa*I fragment of plasmid pMSG (Pharmacia) which carries an MMTV promoter.

For CAT assays the plasmid pMSG-CAT (Pharmacia) was used. This carries the gene coding for chloramphenicol acetyltransferase under the control of the MMTV promoter. The MMTV promoter sequence was removed to produce a promoterless control or replaced by the 5′ *Eco*RI to *Pvu*II fragment (see Fig. 1) carrying the upstream region of the pseudogene. Transfection of CAT-plasmids and transient CAT assays were done as described [6].

2.3. *Other methods*

DNA was isolated, digested with restriction enzymes and transferred for Southern blot analysis using standard procedures [9]. Hybridizations were carried out using either a *tk* cDNA probe [10] or the large *Bam*HI to *Eco*RI fragment of the pseudogene (see Fig. 1). Both were labelled by the random primer method [11].

Preparation of cell extracts from logarithmically grown cells and assays for thymidine kinase were carried out as described previously [12].

3. RESULTS

3.1. *The pseudogene can transform LTK⁻ cells to TK⁺*

We originally wanted to use the mouse *tk* pseudogene as a negative control in experiments in which LTK⁻ cells were transfected with various constructs of the *tk* gene which was followed by selection of TK⁺ clones in the presence of HAT medium [7]. Surprisingly we found that transfection of the pseudogene gave rise to HAT-resistant colonies. The frequency of appearance of such colonies was around 20 clones/μg DNA per 10^6 cells which corresponds to roughly 7% of that obtained by transfection of the *tk* gene of Herpes simplex virus type I (HSV TK), a commonly used control. The specific TK activity of 4 selected HAT-resistant clones (two of which are shown in Table I) was only about 3% of that measured in mouse L cells. Southern analysis showed that these stably HAT-resistant clones carried the pseudogene with copy numbers of 30–50 when the band representing the *tk* pseudogen, present as a single copy, was used as a reference. Fig. 1 shows the results obtained with one clone as an example. Considering that after transfection into LTK⁻ cells the promoterless HSV *tk* gene can give rise to HAT-resistant clones most of which carry the truncated gene in many copies [13], we removed the sequence upstream of the ATG from the mouse *tk* pseudogene (using the *Pvu*II site shown in the scheme in Fig. 1). Transfection of the remaining *Pvu*II-*Bam*HI fragment did not give rise to a single HAT-resistant colony among 10^7 cells. It is also worth mentioning that the transfection frequence obtained in [13] was still lower by nearly an order of magnitude than the one we have obtained with the pseudogene. It was therefore likely that the low TK activity and the HAT resistance of our clones was due to the presence of many copies of the pseudogene with an upstream region having low promoter activity. A comparison of the sequence upstream of the ATG in the pseudogene with the respective one of the gene revealed some homologies. It contains, for instance, a GC rich recognition sequence for transcription factor SP1 [5]. Hence, we added the upstream *Eco*RI to *Pvu*II fragment of the pseudogene (see Fig. 1) in front of the gene for bacterial chloramphenicol acetyltransferase (CAT) on a plasmid and tested the transient CAT activity after transfection into LTK⁻ cells. We found that the upstream sequence of the pseudogene allowed low but significant expression of the CAT gene supporting our interpretation.

The necessity of amplification of the transfected pseudogene for establishment of HAT resistance was proved by an experiment in which the pseudogene was co-transfected with a plasmid carrying the Neomycin resistance gene followed by selection for resistance to Geneticine. When 24 Geneticine-resistant clones were plated on HAT medium, only one grew up after several weeks. This cell line showed about 10% of the TK ac-

Table I

TK activity of various stably transfected cell lines

Cell line	TK activity[a]	(%)
L-cells, control	1350	(100)
LTK⁻ cells, control	12[b]	(1)
LTK⁻ cells transformed to TK⁺ by HSV *tk* gene		
clone 1	109	(8)
clone 2	121	(9)
Pseudogene under own flanking sequence		
clone 1	41	(3)
clone 2	36	(3)
Pseudogene under SV40 late promoter		
clone 1	346	(26)
clone 2	761	(56)
clone 3	295	(22)
clone 4	1321	(98)
Pseudogene under MMTV promoter		
grown in the presence of 1 μM dexamethasone	1150	(85)
grown in the absence of dexamethasone	93	(7)

[a]Specific TK activity is given in pmol TMP formed/mg protein per h. [b]The residual activity in LTK⁻ cells (around 1%) is due to the mitochondrial TK

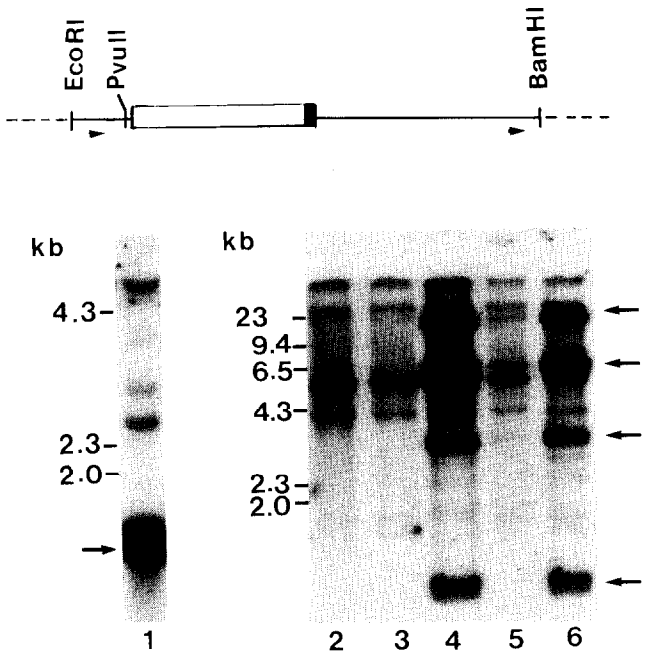

Fig. 1. Genomic Southern blots of cells transfected with the mouse *tk* pseudogene. Top: Schematic drawing of the pseudogene indicating restriction sites used, the open reading frame (box) coding for 177 amino acids of TK and 12 wrong ones (filled part) and the location of the direct repeat sequence (arrows). The total length of the pseudogene is 1475 bp. Below: Southern analysis of *Eco*RI plus *Hin*dIII double digests of DNA from one clone of LTK$^-$ cells stably transformed to the TK$^+$ phenotype with a plasmid containing the pseudogene cloned into the *Eco*RI and *Bam*HI sites of pUC18 (lane 1). Pseudogene sequences are present in highly repetitive form giving a strong band at about 1.5 kb (arrow). The right part shows similar Southern analysis of DNA from a cell line amplifying pseudogene sequences when grown on selective HAT medium. Lane 2: LTK$^-$ cells. Lanes 3–6: LTK$^-$ cells co-transfected with the pseudogene-containing plasmid and a plasmid carrying the Neomycin resistance gene, selection was first on Geneticine. Lane 3: Geneticine-resistant cells grown in normal medium. Lane 4: HAT-resistant cell line selected from Geneticine-resistant cells. Lane 5: the same cell line after transfer to normal medium. Lane 6: the same cell line after transfer back to HAT medium. Arrows point to the bands caused by the amplified pseudogene sequences. The complexity of bands is most likely due to the co-transfection with a second plasmid because the same bands hybridized to a probe containing the *neo* gene.

tivity present in L cells if it was under selection pressure but lost this activity when grown in the absence of HAT. TK activity was restored when cells were set back onto HAT medium. Southern analysis supported the conclusion that this was due to unstable amplification of pseudogene-containing sequences in cells under HAT selection (Fig. 1). The pattern of amplified and reamplified sequences was constant. This, together with the observation that cells under HAT selection exhibited double minute chromosomes which were absent in LTK$^-$ cells and not visible in the transfectant if it was grown in the absence of HAT (Fig. 2) indicates that the pseudogene sequences were present on extrachromosomal structures in this HAT-resistant cell line. Amplification of the pseudogene, therefore, appears to be necessary for these transfectants to produce enough TK to survive HAT selection.

3.2. *The pseudogene can give rise to high TK activity if its expression is under the control of a strong promoter*

In order to find out whether the low transfection efficiency of the pseudogene was due to low promoter activity of the upstream sequence or to the changes in the coding region several plasmids were constructed in which the upstream region (upstream of the *Pvu*II site, Fig. 1) was replaced by the SV40 late promoter or the dexamethasone-inducible MMTV promoter. These constructs had transfection efficiencies close to that obtained with the HSV *tk* gene (around 300 clones/μg DNA per 10^6 cells). Specific TK activities varied among different HAT-resistant clones but could reach values close to those measured in extracts of L cells (Table I). Southern analysis revealed the presence of between one and 5 copies of the pseudogene without correlation between the copy number of the pseudogene and the TK activity present in the cells. The HAT-resistant cell lines were stably TK$^+$ also in the absence of selection pressure. In the case of the pseudogene under control of the dexamethasone-inducible MMTV promoter, the appearance of HAT-resistant clones and of TK activity was dependent on the presence of the glucocorticoid (Table I) supporting the idea that a strong promoter is important for high levels of enzyme expression. Since the pseudogene product lacks the C-terminal 56 amino acids of normal TK and carries instead 12 wrong amino acids, our results imply that the first 177 amino acids suffice for high enzymatic activity.

4. DISCUSSION

There is so far no evidence for activity of a processed pseudogene in mammals. In many experiments we never obtained revertants growing on HAT medium from LTK$^-$ cells, indicating that the pseudogene in situ is inactive and does not amplify under selection pressure. Here we show the first example of the formation of an active product from a pseudogene after transfection. The transfected pseudogene has the ability to amplify and this amplification apparently is necessary for the cells to produce levels of TK sufficient to survive HAT selection. Most likely this requirement is due to the weakness of the promoter rather than to low activity of the truncated and in 3 positions mutated coding region. The strongest support for this assumption comes from experiments which prove that the pseudogene under a strong promoter can give rise to cells which express high levels of TK activity.

It is interesting to consider the base changes the

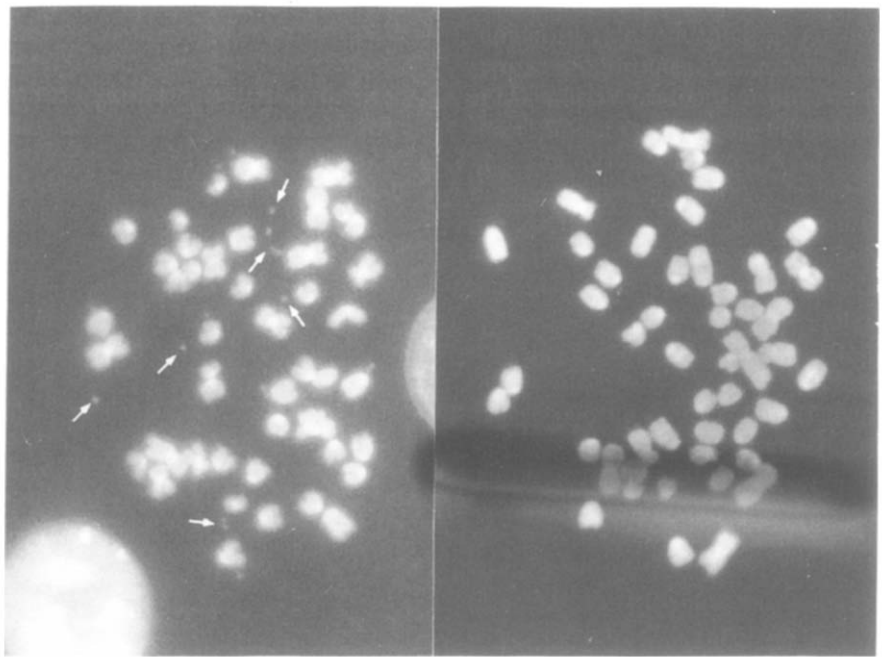

Fig. 2. Karyotype of a cell line transfected with the *tk* pseudogene carrying many copies of the gene in unstable form and exhibiting double minute chromosomes. (A): cells grown in HAT medium; arrows point to double minute chromosomes. (B): cells grown in normal medium.

pseudogene has suffered since its creation and the related question whether the pseudogene was ever active in vivo. A comparison of the sequence of the pseudogene with that of *tk* cDNA revealed the following: (1) There are only 8 base changes in the pseudogene between the ATG and the deletion after amino acid 177, 6 of which are in the third codon position. Only one of these causes an amino acid change which together with two changes in the first codon position accounts for the 3 amino acid changes observed. (2) There are 18 base changes in the remaining part corresponding to the former coding region of the cDNA plus the 3′ non-translated region, which are about equally distributed between first, second and third codon position. Moreover, there are two small deletions and two insertions in this part of the sequence. Together with the fact that the pseudogene is active after transfection this unequal distribution of changes suggests that the deletion of two bases after the codon for amino acid 177 was an early event in the pseudogene, leaving a sequence in the genome which still coded for an active product. This sequence apparently was under selection pressure allowing less frequent changes. Full inactivation of the pseudogene in vivo may have occurred only more recently in evolutionary time perhaps by a rearrangement within the genome.

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