Volume 11 Number 6 1983 Nucleic Acids Research

Elements in the long terminal repeat of murine retroviruses enhance stable transformation by thymidine kinase gene

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Received 22 November 1982; Revised and Accepted 24 February 1983

ABSTRACT

We have investigated the effects of long terminal repeats (LTRs) of murine retroviruses on the frequency of obtaining stable transfectants by the herpes virus thymidine kinase (TK) gene. The results indicate that addition of LTRs enhances the number of TK^+ transformants by 10-20 fold. A 5-12 fold enhancement was also observed when chromosomal DNA from either human or hamster cells was mixed with a plasmid containing LTR sequences and transfected onto LTK^- cells. The LTR sequences involved in the enhancement were localized in the region which contins tandem repeats. All other regions of the LTR did not show any enhancement of stable TK^+ transfectants. The location or the orientation of the enhancer sequences with respect to the TK gene did not exert any influence on the frequency of transformation. The enhancement effect does not appear to be linked to either increased numbers of chromosomal integrations or elevated levels of transcription of the TK gene.

INTRODUCTION

Structural analyses of several cellular and viral genes have identified nucleotide sequences involved in the initiation of transcription. These regulatory sequences are located upstream from the initiation site of mRNA transcripts. A consensus sequence referred to as the "TATA" box is generally located 25 to 35 nucleotides upstream, while another control signal, the "CAT" box, is present approximately 80 nucleotides upstream from the 5'-cap nucleotide (1,2). Mutations introduced in these control elements significantly affect the levels of transcription, both in vivo and in vitro (2-8). For these kinds of transcriptional studies, simian virus 40 (SV40) probably represents the best studied model system, since a number of control elements have been localized in the region around the origin of SV40 DNA replication. In addition to the regulatory elements present upstream from the 5'-termini of the mRNA's, there are sets of 21 bp and 72 bp tandem repeats (9,10). The analysis of several deletion mutants constructed in the 72 bp tandem repeat has shown that removal of precisely one 72 bp repeat does not affect the viability of the virus but deletion into the second 72

bp repeat renders the virus nonviable (2,11-14). Thus, on the basis of these data, several investigators have defined the 72 bp repeat as an essential element for the expression of early SV40 genes.

The control elements involved in the replication of retroviral genomes reside in the long terminal repeat (LTR)(15). Inspection of the sequence of different murine retroviral LTR's reveal the existence of a tandem repeat ranging in size from 50-110 bp about 185 nucleotides upstream from the 5'-cap nucleotide (16-20). Although the tandem repeats in the LTR have no sequence homology with the 72 bp tandem repeats found in SV40, Levinson et al. have shown that the 72 bp tandem repeat present in the Mo-MSV LTR can functionally replace the SV40 72 bp tandem repeat (21). To study the role of the tandem repeats of murine retroviral LTR's in transcription, we have taken a different approach. We have linked LTR sequences to a 3.5 kb DNA fragment containing the herpes simplex virus thymidine kinase gene, and selected transfected LTK$^-$ cells in HAT selection medium (22-24). The number of HAT resistant colonies was counted. Our results indicate 1) Addition of LTR sequences enhances the number of the transfectants by 20-fold; 2) The tandem repeat sequences (either one or both repeats) in the LTR are sufficient to elicit the enhancement effect; 3) No other region of the LTR showed enhancement of the number of TK transfectants; 4) Mixing of plasmid DNA containing LTR sequences with chromosomal DNA from HLFA-3 cells (TK$^+$), followed by transfection of LTK$^-$ cells resulted in 3- to 12-fold greater enhancement of TK transformants. We conclude that the tandem repeats in LTR sequences act as enhancers in transfections with viral and chromosomal thymidine kinase genes. A preliminary account of some of these results has already been given (25).

MATERIALS AND METHODS

Cell culture and transfections. Mouse LTK$^-$ cells (26,27) were grown in DME (Gibco) plus 10% calf serum (Irvine Scientific); Chinese hamster (CHO TK$^-$) obtained from R. Hofmann (28) and HLFA3 (29) cells were grown in the same medium with 10% fetal calf serum supplemented with non-essential amino acids. HeLa cells were grown in DME with 10% fetal calf serum. Cells were transfected using the calcium phosphate precipitation technique as previously described (22,30). When carrier was used it was DNA from the recipient cell line (LTK$^-$ or CHO TK$^-$). The calcium phosphate DNA precipitates were left on the cells for approximately 20 h,

and selection for TK^+ cells was started 48 h after application of the DNA, using the appropriate medium supplemented with HAT (1×10^{-4} M hypoxanthine, 4×10^{-6} M aminopterin and 5×10^{-5} M thymidine). Surviving colonies were counted between 10 and 15 days after plating the cells, by visual inspection. This excluded any colonies with fewer than approximately 50 cells. Colony numbers usually increased after this stage but these numbers were not used because of the possibility of secondary colonies.

Individual isolated colonies were picked using a micropipet, and grown under HAT selection.

Recombinant DNA techniques. The general procedures for molecular cloning have previously been described(31). The details of construction of various plasmids are given in the text (Figs. 1 and 2). In general, DNA fragments were fractionated on agarose gels and DNA from appropriate bands eluted with the glass powder procedure as described (17).

DNA and RNA preparations and their analysis. Approximately 10^8 cells were lysed in 30 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5% Triton X-100, pH 8.3 (P2 buffer). The nuclei were spun down at 9,000 g and DNA purified by conventional means (32). The supernatants were used to prepare cytoplasmic RNA: the sample was made 0.5% in SDS, 10 mM in EDTA, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1) and ethanol precipitated. DNA was digested to completion with Hind III, electrophoresed on 0.8% agarose gels and blotted to nitrocellulose by the method of Southern (33) using acid pretreatment to increase transfer of higher molecular weight materials. Filters were hybridized to ^{32}P nick-translated (34) denatured probes in 6x SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.4) plus 5x Denhardt's additives (35) and 250 ug/ml sonicated denatured herring sperm DNA (Sigma) at 68° C in the presence of 10% dextran sulphate (32).

RNA was glyoxylated, electrophoresed on 1.2% agarose gels and blotted to nitrocellulose as described by Thomas (36). RNA filters were hybridized as for the DNA filters, in the presence of 0.1% SDS and 50 ug/ml yeast RNA. After hybridization the filters were washed and exposed to Kodak XAR 5 X-ray film.

RESULTS

Construction of plasmids

A number of recombinant plasmids containing genome length viral DNA or only LTR sequences or portions of LTR sequences were constructed. The structure of various recombinant plasmids is shown in Figs. 1 and 2.

1) pMSV-TK-7: The unintegrated form of Mo-MSV DNA was molecularly cloned in bacteriophage Charon 21a and the insert was subsequently subcloned in the unique Hind III site of pBR322 (pMSV-12)(31). The plasmid pMSV-12 has two long terminal repeats separated by 321 bp(17). Additionally, the 5' LTR is inverted from the expected orientation (Fig. 2 in ref 17). The unique Bgl II site in the pMSV-12 was used to introduce a 3.5 kb Bam HI fragment containing the herpes virus thymidine kinase (TK) gene(37).

2) pMSV$_i$ -1L-TK: Mo-MSV proviral DNA obtained from G8 cells (NIH/3T3 cells infected with and producing Mo-MSV clone 124) was molecularly cloned in bacteriophage Charon 30 and the 7.8 kb Eco RI insert containing the entire viral genome was subcloned in pBR322 (38). The TK gene contained in the 3.5 kb Bam HI fragment was introduced in the unique Bgl II site of Mo-MSV viral DNA.

3) pLTR-10: The Hind III-Pst I fragment (see pMSV-TK7) containing the two LTR's present in pMSV-12 was isolated and treated with S1 nuclease to create blunt ends. Synthetic linkers containing Eco RI sites were added onto this fragment and cloned in the unique Rl site of plasmid pBR325.

4) pLTR-TK-101: The Rl insert from plasmid pLTR-10 was transferred to the Rl site of pBR322, followed by insertion of the 3.5 kb TK-Bam HI fragment in the unique Bam HI site of pBR322.

5) pSV7: The Rl insert containing the LTR was transferred to the Rl site of the plasmid,pSV2 (39) which contains the SV40 origin sequences, including the 72 bp repeats but cannot make T antigen.

6) Subclones of portions of LTR: Various portions of the LTR shown in Fig. 2 were subcloned. The restriction fragment containing a portion of the LTR was extended with a stretch of dC residues and annealed to Cla I digested and (dG)-tailed pBR322. The 3.5 kb Bam HI fragment containing the herpes TK gene was then inserted in the unique Bam HI site of the plasmid pBR322. The following subclones were made:

a) pTR-128-8: A 128 nucleotide long Sau3A-Xba I fragment containing one 85 bp tandem repeat and an additional 43 nucleotides was

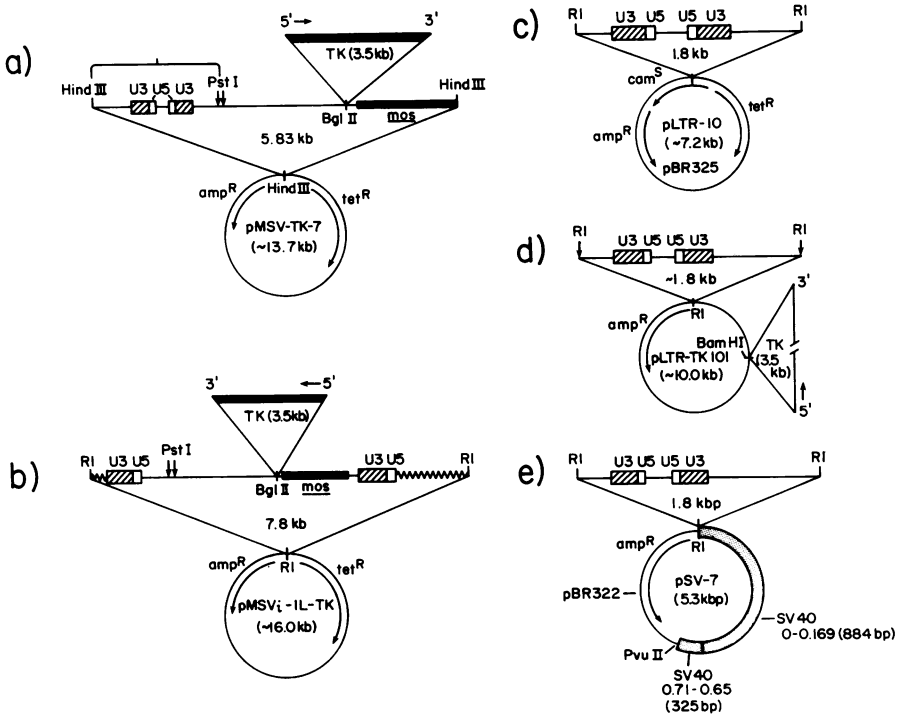

FIG. 1. Recombinant plasmids containing total MSV or two LTR sequences.
U3, unique 3' end sequences of viral genome; U5, unique 5' end sequences of the viral genome; mos, transforming gene of Mo-MSV (15).

isolated from the LTR of plasmid pMLV-1D (Fig. 2a). This plasmid contains only one copy of the repeat sequences in the LTR (40).

b) pTR-TK-128-8: The plasmid pTR-128-8 containing the 3.5 kb TK fragment.

c) pTR-203-9, pTR-203-26: Digestion of pMLV-1A (containing 2 tandem repeats in its LTR;17) with restriction endonuclease Xba I and Sau3A generates a fragment of 203 nucleotides containing two tandem repeats. The orientation of the insert with respect to the Eco RI site of pBR322 is reversed in the two clones.

d) pTR-TK-203-9, pTR-TK-203-26: The 3.5 kb herpes virus TK Bam HI fragment was introduced in the Bam HI site of the two plasmids.

e) pPH-TK: This plasmid contains a fragment of 142 nucleotides representing the 5'-end of the genome (U5 + R) [obtained from plasmid

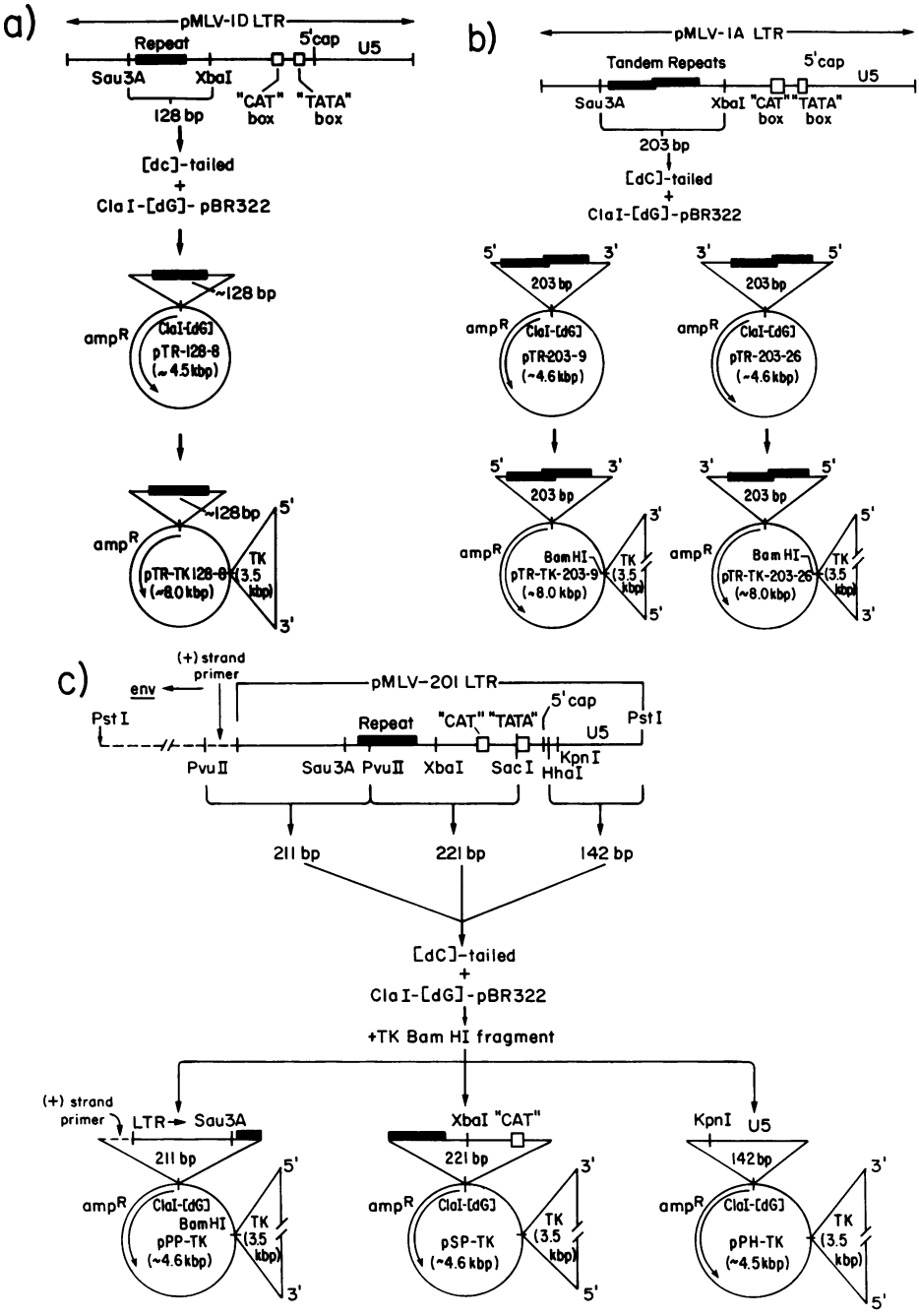

FIG. 2. Recombinant plasmids containing portions of LTR sequences.

pMLV-201 (41)]and the 3.5 kb fragment containing the TK gene.

f) pSP-TK: The DNA from plasmid pMLV-201 (17) containing the Mo-MLV LTR was cleaved with Sac I and Pvu II. This fragment contains sequences adjacent to the putative promoter signal and a portion of the 85 bp tandem repeat. The plasmid also has the TK gene.

g) pPP-Tk: This plasmid contains a fragment containing a portion of 85 bp repeat and adjacent sequences from the 5'-end of the LTR. The TK gene was introduced in the Bam HI site of pBR322 (Fig. 2c).

Enhancement of TK transformants.

Table I summarizes the results obtained using a number of different recombinant clones containing the TK gene. Two sets of experiments are shown, one where the number of TK transfectants was high (A, 5 TK colonies/ng of pTK/10^6 cells) and the other where the number of TK transfectants is low (B, 1 TK colony/5 ng of pTK/10^6 cells). In both cases, results obtained with and without carrier DNA are shown. The extent of enhancement of TK colonies obtained without the carrier DNA is tabulated. All recombinant clones containing one or two repeat sequences present in the LTR show 10 to 20-fold enhancement of TK transfectants. Recombinant TK clones containing other regions of the LTR [$U5^+$ R regions (pPH-TK)]; sequences adjacent to the presumptive "TATA" box and encompassing a portion of the repeat sequences (pSP-TK), or the 5'-end of U3 sequences (pPP-TK) did not show any enhancement of transformation by the TK gene.

Mixing enhancer sequences with TK gene.

Table 2 shows an experiment where plasmid pLTR10 containing the MSV LTR's (see Fig. 1), was mixed with pTK. A five-fold increase in the number of colonies that became HAT resistant (i.e., TK^+) was observed compared to a 12.5-fold increase for pMSV-TK7 in which the LTR's were covalently linked to the TK gene (see Fig. 1). Also shown is an enhancement of five-fold achieved with pSV7 which has both MSV LTR's and the SV40 72 base pair repeat enhancer sequence. Thus in this situation the effects of two types of enhancers are not additive. These results for mixing in MSV LTR sequences were reproducible, with the enhahcement varying from 3-12 fold with the addition of 2 ug of enhancer plasmid. We also attempted to enhance the number of transformed colonies in HAT media by pSV2-gpt, pSV3-gpt and pSV5-gpt (39) in mouse LA9 cells by adding enhancer plasmid. No enhancement was seen, presumably because the plasmids are already under the influence of the SV40 72 base pair

TABLE 1

Plasmid	A			B		
	Number of TK^+ colonies (high)		Fold Enhancement	Number of TK^+ colonies (low)		Fold Enhancement
	5ng plasmid + 20 µg carrier	5µg plasmid (no carrier)	(no carrier)	5ng plasmid + 20 µg carrier	2µg plasmid (no carrier)	(no carrier)
PBR 322	0	0	-	0	0	-
PTK	24	111	1	1	6	1
pMSV-TK7	202	>2,000	>18	10	118	20
$pMSV_i$-IL-TK	260	>2,000	>18	10	113	19
pLTR-TK-101	140	1,200	11	3	108	18
pTR-TK-128-8	160	>2,000	>18	21	55	9
pTR-TK-203-9	170	>2,000	>18	22	111	19
pTR-TK-203-26	330	720	7	9	59	10
pPH-TK	12	108	0.97	2	6	1
pSP-TK	26	99	0.89	1	5	0.83
pPP-TK	13	72	0.65	2	4	0.67

Table 1. Transfection frequencies into LTK^- cells of various TK plasmids covalently linked to MSV sequences. Two typical experiments, A and B, are shown. Frequencies were 10-20 fold higher in A than B for unknown reasons, and the two experiments represent the extremes of the spectrum of frequencies which we experienced for individual plasmids. Nevertheless, relative frequencies between plasmids did not change. Plasmid structures are described in Fig. 1. Fold enhancements listed are for the carrier-free experiments. All experimental numbers are the average of the number of colonies on duplicate plates.

TABLE 2

Plasmid	Number of TK^+ colonies per plate (av. of 2)	Fold Enhancement
pBR 322	0	-
pTK	2	1
pMSV-TK7	25	12.5
pTK + pLTR-10 (2µg)	10	5
pTK + pSV7 (2µg)	10	5

Table 2. Transfection frequencies into LTK^- cells of pTK with or without added enhancer plasmids.
Five ng of plasmid plus 20 ug carrier DNA were added per plate. In addition, 2 ug of pLTR10 or pSV7 per plate were added as shown. Numbers shown are the averages of two plates.

repeat and are maximally enhanced.

Effect of LTR enhancer sequences on chromosomal DNA transfections.

We next determined if the effect of mixing enhancer sequences also applied to transfection by chromosomal DNA. Table 3 shows results of experiments in which human (HeLa) and hamster (HLFA-3) total chromosomal DNA were used as the source of TK gene. A 3- to 10-fold enhancement was observed with either pLTR10 (containing MSV LTR's) or pTK-203-26 and pTK-128-8 (containing two or one repeat in LTR, respectively). En-

TABLE 3

Plasmid	Number of TK+ colonies HeLa DNA	Fold Enhancement	Number of TK^+ colonies Hamster DNA	Fold Enhancement
pBR 322	8	1	1	1
pLTR-10	50	6.25	21	10.5
pTR-203-26	28	3.5	23	11.5
pTR-128-8	25	3.1	-	-

Table 3. Transfection frequences into LTK^- cells of chromosomal TK with or without enhancer plasmids.
Twenty ug of either human (HeLa) or Chinese hamster (HLFA3) DNA's plus 2 ug of the plasmids shown were added per plate. Numbers for the HeLa DNA experiment are the totals from 8 plates and for the Chinese hamster DNA are the average of two plates.

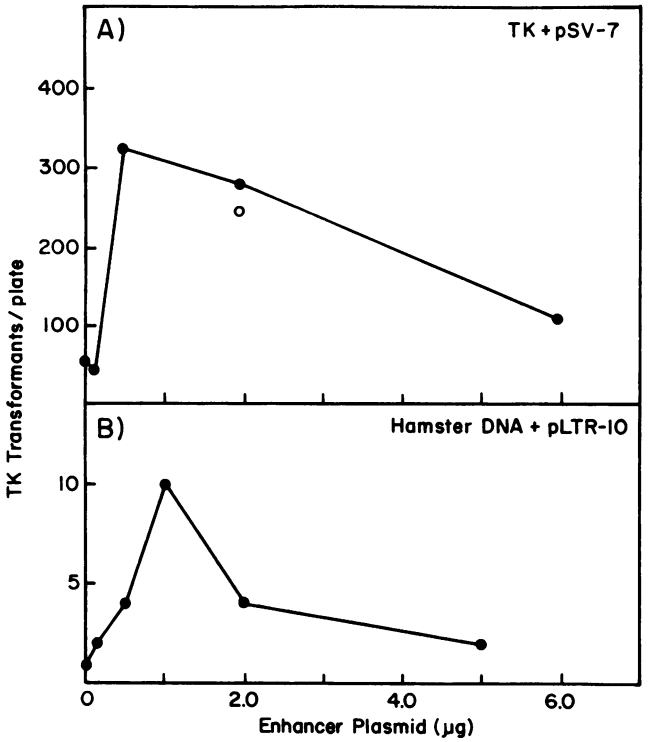

FIG. 3. Titration of enhancer sequences for ability to increase transfection frequencies. LTK$^-$ cells were transfected with a constant number of TK genes in the presence of varying amounts of enhancer plasmids, as described in Materials and Methods, and the resulting HAT resistant TK$^+$ colonies counted. Each point is the average of two plates. Top: 50 ng intact pTK (herpes TK gene) plus 20 ug LTK$^-$ carrier DNA per plate in the presence of intact pSV7. The open circle at 2 ug of pSV7 represents an experiment using Bam Hl cut (linearized) pSV7. Bottom: 20 ug Chinese hamster (HLFA3, TK$^+$) chromosomal DNA plus intact pLTR10.

hancement, however, seemed to be higher with hamster (ca. 10-fold) than human (3-7 fold) DNAs. To establish the optimum dose of enhancer sequences, we performed ex-periments in which increasing amounts of plasmid containing either pSV7 (SV40 enhancer sequences) or pLTR10 were mixed with pTK or hamster chromosomal DNA prior to transfection. Results shown in Figures 3A and B indicate an optimum of 1-2 ug of enhancer plasmid per 20 ug of carrier + pTK or 20 ug of hamster chromosomal DNA.

We also tried to enhance the transfection of mouse LA9 HPRT$^-$ cells

TABLE 4

Plasmid	Number of TK^+ colonies	Fold Enhancement
pBR 322	0	-
pTK	1	1
pMSV-TK7	26	26

Table 4. Transfection into CHO TK^- cells.
100 ng of plasmid plus 20 ug carrier DNA were added per plate. The numbers shown are the average of two plates.

with chromosomal human HPRT using human placental DNA by mixing with pLTR10. No enhancement was observed in two experiments (data not shown).

Enhancement in other cell lines.

Table 4 shows that enhancement of TK transfection is not confined to LTK^- cell lines, but also can be observed when transfection is performed with Chinese hamster ovary (CHO) TK^- cell lines. A 26-fold enhancement of the transformed colonies is observed when pMSV-TK7 is used. Even

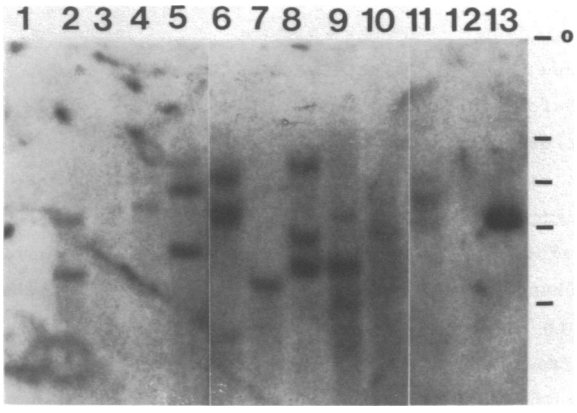

FIG. 4. Southern blot of DNA from pTK and pMSVTK7 transfected LTK^- cells. Hind III digested DNA (12 ug per lane) was electrophoresed, blotted and hybridized to the purified, ^{32}P-labeled 3.5 kb Bam Hl TK fragment as described in Materials and Methods. Lane 1, LTK^- DNA. Lanes 2-6, DNA from five different pTK transfected cell lines. Lanes 7-11, DNA from five different pMSV-TK7 transfeced cell lines. Lane 12, LTK^- DNA. Lane 13, Hind III cut pTK (5 gene equivalents). The markers on the right side are, from the top: the origin, and the /Hind III markers at 23.4, 9.6, 6.6 and 4.3 kb (54).

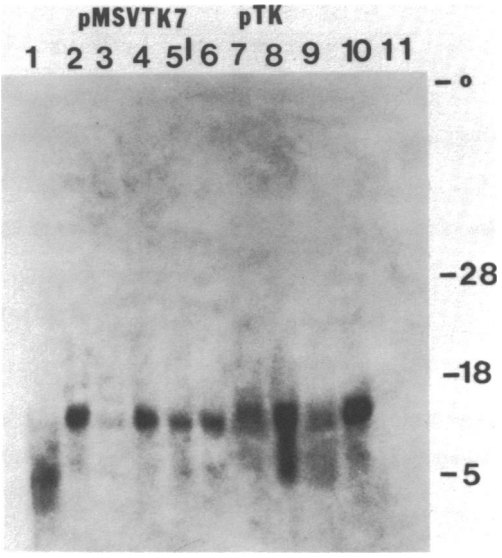

FIG. 5. Northern blot of RNA from pTK and pMSV-TK7 transfected LTK⁻ cells. Total cytoplasmic RNA (25 ug per channel) was electrophoresed, blotted and hybridized to pTK as described in Materials and Methods. Lanes 1-5, RNA from five different pMSV-TK7 transfected cells. Lanes 6-10, RNA from five different pTK transfected cells. Lane 11, LTK⁻ RNA. The markers on the right side are, from the top: the origin, 28S, 18S and 5S RNA.

though the efficiency of TK transformation in CHO TK⁻ cell lines is generally 10-100 fold lower than that observed in LTK⁻ cell lines, the fold enhancement observed with LTR enhancer sequences is similar (about 20-fold) in both cases.

Mechanism of enhancement.

We considered the two most likely possibilities to explain the mechanisms of enhancement: (a) increased integrations of the number of plasmid DNA's into the genome; (b) increased efficiency of RNA synthesis in the stable transfectants, allowing increased survival of transient transfectants in the early stages of transfection. We tested the first possibility by examining the number of integrated copies of the gene in pTK and pMSV-TK7 transfectants by Southern blot analysis (33)(Fig. 4). Approximately equivalent situations are seen in both types of transfectants with 1-5 copies of the TK gene/cell as is normal for pTK transfections (42). Thus there is no apparent increase in the number of integrated TK genes per cell in lines transformed with LTR containing

plasmids. This is also the case for the hamster cells (data not shown).

We tested the possibility of an increased number of stable transfectants being linked to increased transcription by measuring the relative levels of TK RNA transcripts in pTK and pMSVTK7 transfected cells. Figure 5 shows an autoradiograph of the total cytoplasmic RNA isolated from both types of cells hybridized to ^{32}P labeled 3.5 kb TK gene fragments. The levels of TK specific RNA transcripts in the pMSV-TK7 transfectant are not higher than in the pTK transfectants. In addition, a majority of the transcripts are the same size in both types, ruling out any significant transcription initiating in the LTR promoter (see Fig. 1).

DISCUSSION

Definition of an enhancer element.

Recently a number of small (100-200 base pairs) DNA sequences from various viral genomes have been shown to have an "enhancer" activity on either transient or stable transfection frequencies in tissue culture cells (13,14,21). The archetype has been the 72 base pair repeat from the late side of the SV40 origin (9,10) but sequences from polyoma (43) and bovine papilloma (44) viruses have also been shown to be effective. Originally it was shown that although the SV40 72 base pair repeats were not required to obtain efficient transcription in in vitro extracts, deletion of these repeats rendered the resulting DNA completely non-infectious (43). However, one complete copy of the 72 base pair repeats was sufficient to make the SV40 DNA completely viable. In addition, it has been shown that the 72 base pair non-homologous repeats from MSV LTR sequences (the equivalent fragment to the one in pTR-203-9 in this paper) can substitute for the SV40 repeats to give a viable virus (21). These enhancer sequences have thus been defined in three ways: (i) ability to increase the level of transient transcription (1-3 days) after transfection (14,43-45,46); (ii) requirement for viability in a virus (13,21); (iii) ability to increase the number of stable transfectants using a selectable marker such as TK. The first of these has been the most widely used definition, since it is a shorter experiment than (iii), and (ii) can only be carried out in a limited number of situations. Thus it is not yet established whether all these are necessary features of the action of enhancer sequences.

We have been able to show that when the herpes thymidine kinase gene

is covalently linked to the cloned Mo-MSV viral DNA, an approximately 20-fold enhancement is achieved in the number of stably transfected HAT resistant colonies in LTK$^-$ and CHOTK$^-$ cells, per TK gene. The sequences responsible for this enhancement have been narrowed down, firstly to the MSV LTR sequences, and finally the 75-85 base pair repeats, only one of which was necessary for the effect. The position and the orientation of the enhancer with respect to the TK promoter seemed irrelevant to the effect, as has been found with other transcription enhancers (45).

Enhancers can affect transfection efficiency in "trans"

Interestingly, we found a reduced but significant enhancer effect (3-12 fold) merely by mixing the enhancer sequences with the TK gene into the transfection mix. An explanation for this "trans" effect is that when the transfection takes place, the incoming DNA is ligated together into fairly large DNA molecules (47), so that if sufficient enhancer sequences are present, it is likely that one will be near the gene for which selection is being applied, in effect becoming "cis". In the case of pMSV-TK7, even though the enhancer sequences are present over 5 kb away from the TK promoter, they are still very effective (Fig. 1, Table 1). It has previously been shown that a DNA fragment containing the transforming gene (*mos*) of the Mo-MSV can be activated to transform fibroblasts in vitro if mixed with a plasmid containing LTR sequences (48).

The ability of LTR sequences to enhance transformation frequencies of TK genes in a nominal "trans" configuration suggested that they might perform the same function with chromosomal genes. A 3-12 fold enhancement of stable TK transfections was obtained with human and hamster chromosomal DNA (Table 3). However, we were unable to increase transfection frequencies with human HPRT transfected into mouse LA9 cells. We attribute this to the possible existence of endogenous enhancers close to the HPRT gene, so that no further enhancement can be achieved or the efficiency of the HPRT promoter cannot be enhanced. The apparent difference in the ability to enhance the human (3-7 fold) and hamster (5-12 fold) TK genes could also be due to linked enhancer sequences near the human gene which sometimes retain covalent linkage, or which are rather ineffective over a long distance. Compatible with this concept is the recent observation that in transient transfection assays, some members of the human globin cluster can be enhanced by SV40 repeats while other cannot (49). It thus appears that the effect of

enhancer sequences is not additive. Consistent with this notion is the observation that SV40 enhancer sequences (72 bp repeats) were unable to augment the enhancement when MSV LTR sequences were already present (Table 2). Conversely, the enhancer element in MSV LTR was not capable of increasing the enhancement by SV40 enhancers, thus leading to the speculation that they operate by a common mechanism.

Mode of enhancement

We investigated directly two possible mechanisms for the observed enhancement: (1) an increased ability to integrate in the host genome; (2) an increased transcription of the TK genes in stable transfectants. As was seen in the Results section (Figs. 4 and 5), neither of these appears to be direct explanations of the effect. On the face of it, this was unexpected since the MSV LTR enhancers do not appear to increase the transcription, while the SV40 repeats, for which they can substitute to maintain virus viability, do act at least partly by increasing transcription in transient transfection (50). One possible explanation may be linked to the fact that, of the many cells transiently transfected in an experiment, only a few become stably transfected and survive to make colonies. It should, however, be noted that transcriptional levels in transient transformants were not assayed in this study.

The precise mechanism by which enhancer elements exert their influence remains obscure. We suggest that in the case of an enhanced number of TK transfectants observed here, two mechanisms might operate. Firstly, the enhancer elements may increase the overall number of transiently transfected cells which enhance the probability of obtaining larger numbers of stably transfected cells. Alternatively, the presence of enhancer elements facilitates the process by which transient transfectants are converted to stable transfectants. The enhancer elements may aid in the process of integration of transfected DNA into host chromosomes.

In a recent publication, Joyner et al. (51) have shown that covalent linking of SFFVp LTR to the herpes virus TK gene did not show any enhancement of TK transformed colonies. The nucleotide sequence of SFFVp LTR is 86% homologous to the LTR of Mo-MLV and contains one copy of the repeat sequence (52). However, the U5 region of SFFVp LTR and Mo-MLV LTR is over 91% homologous (52). In this connection, it is interesting to note that the Rous associated virus (RAV-0) LTR is unable

to enhance the number of TK transfectants whereas the Rous sarcoma virus (RSV)LTR shows an enhancement of 20-60 fold (P. Luciew, J. M. Bishop, H. E. Varmus and M. Capecchi et al., personal communication). The RAV-0 LTR is about 52 nucleotides shorter than the RSV LTR (53) and most of the non-homology is in the U3 region. Thus it appears that two rather similar LTRs exert different influences on the enhancement of stable TK transfectants.

ACKNOWLEDGMENTS

We thank Dr. C. Van Beveren for help in constructing many plasmid DNAs and Dr. L. Enquist for providing the herpes virus thymidine kinase gene.

This work was supported by U. S. Public Health Service grants No. GM-28223 and CA-24288 (T. F.) and CA-21408, CA-2-6561 and ACS MV-100B (to I.M.V.).

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