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Location and Function of Retroviral and SV40 Sequences That Enhance Biochemical Transformation after Microinjection of DNA

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Summary

Biochemical transformation of thymidine-kinase-deficient (TK⁻) mouse L cells is enhanced 20 to 40 fold when microinjected plasmid DNA contains regions of the genomes of Rous sarcoma virus or simian virus 40 in addition to the complete herpes simplex virus *tk* gene, irrespective of the orientation and location of the enhancer. The enhancing sequence of RSV DNA has been localized to 143 bp that include 88 bp at the 5′ end of the long terminal repeat; the enhancing segment of SV40 DNA lies 42–276 bp upstream from the initiation site for early transcription. The RSV enhancer does not provide a favored integration site within microinjected plasmids and does not affect the frequency of integration into host cell DNA. When microinjected cells were grown into cell lines in nonselective medium, few cells containing integrated HSV *tk* without RSV DNA could grow in selective medium. In contrast, over 80% of cells from lines containing the HSV *tk* gene linked to the RSV enhancer could grow in selective medium. Thus the RSV enhancer affects the expression rather than the integration of the *tk* gene.

Introduction

Retroviruses provide useful systems for elucidating the mechanisms by which eucaryotic cells control gene expression since they insinuate themselves into the life cycle of a cell by integration into host chromosomes (Bishop, 1978; Varmus and Swanstrom, 1982). An integrated retrovirus genome, the provirus, contains the viral genes flanked by long terminal repeat (LTR) sequences covalently linked to cell DNA (Hughes et al., 1978; Sabran et al., 1979). The LTR sequences contain control elements that regulate viral gene expression as well as sites for precise joining of viral DNA to host cell DNA (Varmus, 1982). Encoded in the LTRs are signals for initiation of transcription and for processing of viral transcripts by addition of poly(A) tails. In addition to controlling viral gene expression, the sequence encompassing the LTRs of some avian retroviruses appears to play a role in activating expression of a cellular gene in ALV-induced leukosis (Payne et al., 1981; Neel et al., 1981; Hayward, Neel, and Astrin, 1981; Payne, Bishop, and Varmus, 1982). In some tumors, an activated oncogene is positioned so that the viral LTR cannot function as its promoter (Payne et al., 1982), suggesting that the viral DNA contains a sequence that enhances expression from heterologous promoters, as described in other systems (see Discussion).

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The availability of molecular DNA clones of the RSV genome has facilitated investigations of the function of LTR sequences in integration and regulation of viral gene expression (DeLorbe et al., 1980). However, when cultured mammalian cells are transformed with various forms of retroviral DNA applied as coprecipitates with calcium phosphate (Graham and Van der Eb, 1973), the LTRs are not directly involved in integration into the host cell genome (Chang et al., 1980; Copeland et al., 1981; Kriegler and Botchan, 1983; P. A. L., J. M. B., and H. E. V., unpublished data). For introducing DNA into tissue culture cells, needle microinjection with glass micropipettes is an alternative to transfection (Graessmann et al., 1979; Capecchi, 1980). Controlled numbers of DNA molecules can be microinjected directly into the nuclei of viable tissue culture cells, and biochemical transformation of thymidine-kinase-deficient (TK⁻) mammalian cells by the cloned HSV *tk* gene (Wigler et al., 1977) provides a convenient assay for assimilation and expression of cloned DNA in recipient cells (Capecchi, 1980).

We examined the consequences of microinjecting plasmid DNA that contains RSV sequences into the nuclei of TK⁻ mouse L cells. Plasmids with both the HSV *tk* gene and the region of the cloned RSV genome encompassing the RSV LTR converted about one fifth of the microinjected TK⁻ mouse L cells to stable TK⁺ transformants, a frequency 20 to 40 times that observed with plasmids containing the HSV *tk* gene alone. The enhanced level of TK transformation is similar to that previously obtained with HSV *tk* plasmids harboring a region of the SV40 genome (Capecchi, 1980). The RSV and SV40 sequences responsible for enhancement of TK transformation have been further defined and compared. For each virus, a unique sequence approximately 100–250 bp upstream from a Goldberg–Hogness TATA box has been demonstrated to possess enhancing activity. By examining the integration and expression of DNA in microinjected cells, including cells propagated under nonselective conditions, we have found that the RSV enhancer region functions by activating the expression of the integrated HSV *tk* gene in recipient cells, rather than by facilitating integration or stabilization of the injected DNA.

Results

Enhancement of TK Transformation by RSV DNA

To study the effect of retroviral sequences upon integration and the regulation of transcription, we have microinjected tissue culture cells (TK⁻ mouse L cells) with linearized forms of plasmid DNAs containing both the HSV *tk* gene and a 1022 bp region of the RSV genome encompassing

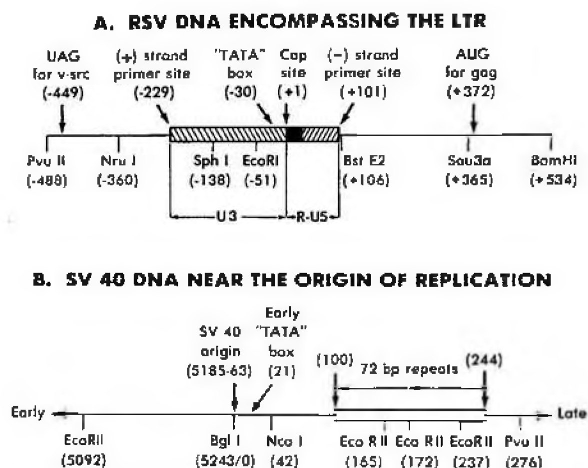

Figure 1. Regulatory Regions of the RSV and SV40 Genomes

(A) The site for initiation of RSV RNA (cap site at +1) divides the LTR into the U3 sequence (229 bp) and the R-U5 sequence (101 bp). R is a 21 bp sequence (+1 to +21) found at the ends of RSV RNA. U3 and U5 are unique to the 3′ and 5′ ends of viral RNA. A "TATA" sequence is present within U3 at positions −24 to −31. Shown also are the regions upstream and downstream from the LTR, including the positions of the termination codon for the viral transforming gene (*v-src*), the initiation codon for the *gag* gene, and the priming sites for synthesis of plus and minus strands of DNA. The sequence of the RSV LTR is presented in Swanstrom et al. (1981); the sequences upstream from the LTR are reported by Czernilofsky et al. (1980) and those downstream by Swanstrom et al. (1981).

(B) The segment of SV40 genome shown includes the origin of SV40 DNA replication, the "TATA" box for early gene expression, and the 72 bp direct repeats. The sequence of this region of SV40 DNA is presented by Fiers et al. (1978).

The number assigned to each restriction site was determined from the midpoint of the recognition signal; for 4- and 6-base sites, the lower number at the midpoint was used.

Table 1. Transformation Frequencies Obtained by Microinjecting Plasmid DNA Containing the HSV tk Gene and RSV DNA

Structure of Microinjected DNA	Number of TK^+ Colonies per 10^3 TK^- Cells Injected
pTK-A (Sa, Bg/Ba, Bg, Ba, Sa)	12
pTK-B (Sa, Bg/Ba, Bg, Ba, Sa)	6
pAV-11/TK-A (Sa, Bg/Ba, Ba, Bg, Ba, Sa)	226
pAV-11/TK-B (Sa, Bg/Ba, Ba, Bg, Ba, Sa)	206
pAV-11/TK-AA (Sa, Bg/Ba, Bg, Bg/Ba, Ba, Sa)	240
pAV-11/TK-BB (Sa, Bg/Ba, Bg, Bg/Ba, Ba, Sa)	196

The heavy lines denote RSV, the medium lines HSV *tk*, and the light lines plasmid sequences. Arrows indicate the normal direction of transcription from RSV and HSV *tk* promoters. The hatched boxes show the U3 and R-U5 portions of the RSV LTR as in Figure 1; the closed circle is the HSV *tk* promoter. pTK-A and pTK-B contain a 3.4 kb Bam HI fragment of HSV DNA with the entire *tk* gene in either orientation in pBR328. pAV-11/TK-A and pAV-11/TK-B represent both orientations of the 3.4 kb HSV *tk* DNA fragment cloned into the Bam HI site of pAV-11, a derivative of pBR328, containing a 1 kb segment of RSV DNA. Similarly, pAV-11/TK-AA and pAV-11/TK-BB represent both orientations of the 3.4 kb HSV *tk* DNA fragment cloned into the Bgl II site of pAV-11. The designations for restriction endonucleases are as follows. Ba: Bam HI. Bg: Bgl II. Sa: Sal I. The transformation frequency is expressed as the number of TK^+ colonies per total number of microinjected TK^- L cells (see Experimental Procedures). Each cell received approximately 20 molecules of plasmic DNA that had been linearized with Sal I.

the LTR (Figure 1). In general, about 0.5% to 1.0% of cells microinjected with plasmids pTK-A and pTK-B, which lack RSV DNA, acquired the ability to form colonies in HAT medium (Table 1). A 20 to 40 fold higher level of TK transformation was observed with plasmids containing the RSV DNA fragment, regardless of transcriptional orientation of the HSV *tk* gene (pAV-11/TK-A and pAV-11/TK-B in Table 1). A similar effect can be obtained with the other possible orientations of the RSV sequence relative to the HSV *tk* gene (pAV-11/TK-AA and pAV-11/TK-BB in Table 1). Thus the RSV sequence can enhance the frequency of TK transformation when it is located about 1 kb upstream and oriented toward the HSV *tk* gene; upstream and oriented away from the HSV *tk* gene; about 2 kb downstream and oriented toward the HSV *tk* gene; and downstream and oriented away from the HSV *tk* gene. A similar level of TK^+ transformants was observed if the HSV *tk* plasmids contained one or two LTRs (M. R. C. and P. A. L., unpublished data).

Efficient Transformation by Plasmids with RSV DNA as a promoter for HSV *tk*

In the experiments shown in Table 1, RSV DNA enhanced the efficiency of TK transformation irrespective of its position within microinjected plasmids containing a complete HSV *tk* gene. We observed a similarly high level of TK transformation when cells were microinjected with plasmids constructed with a fragment containing the RSV LTR and an HSV *tk* gene deprived of its own promoter (Table 2). In this case, efficient transformation was obtained only when the RSV DNA and the promoterless HSV *tk* gene were aligned in the same transcriptional orientation (pAV-2/ΔTK-A); very few transformants were produced when the microinjected plasmids contained RSV and HSV *tk* DNA in opposite orientations (pAV-2/ΔTK-B) or the promoterless HSV *tk* gene alone (pΔTK) (Table 2).

These results suggested that the RSV DNA supplies a promoter that functions efficiently to express HSV *tk* in cells receiving pAV-2/ΔTK-A. This conclusion is reinforced by analysis of polyadenylated RNA from transformed cell lines. The native HSV *tk* mRNA is 1.4 kb in cells transformed with pTK (Figure 2, lanes A and I). In a cell line transformed by pAV-1/ΔTK-A, both the size of the dominant HSV *tk* mRNA (1.7 kb, Figure 2, lane B) and its

Table 2. Retroviral Sequences Containing an LTR Function as Promoter and Enhancer: Transformation Frequencies Obtained by Microinjecting Plasmids Containing the HSV *tk* Gene Lacking Its Own Promoter

STRUCTURE OF MICROINJECTED DNA	NUMBER OF TK^+ COLONIES PER 10^3 TK^- CELLS INJECTED
pΔTK (H3 Bg/Ba … Pv Ba H3)	0.2
pAV-2/ΔTK-A (H3 Ba Pv … Bg/Ba … Pv H3)	226
pAV-2/ΔTK-B (H3 Bg/Ba … Pv Ba … Pv H3)	0.2

pΔTK contains a 2.6 kb Bgl II–Bam HI fragment of HSV DNA, with the *tk* gene lacking its native promoter, in pBR322. pAV-2/ΔTK-A and pAV-2/ΔTK-B were made by cloning the same HSV *tk* DNA fragment in both orientations into the Bam HI site of pAV-2, a derivative of pBR322 containing the region of RSV DNA from −488 to +365 (see Figure 1). The heavy lines indicate retrovirus, the medium lines, HSV *tk*, and the light lines plasmid vector sequences. Retrovirus LTR sequences are shown as hatched boxes denoting U3 and R-U5 sequences. The solid arrows denote the direction of normal transcription of retrovirus DNA and the broken arrows of HSV *tk* DNA.

composition (HSV *tk* sequences linked to RSV U5 sequences but not U3 sequences, unpublished results) are consistent with the proposal that transcription is initiated within the RSV LTR, near or at the normal cap site. In a cell line transformed by pAV-2/ΔTK-B, four prominent RNA species were observed (Figure 2, lane C), all larger than the transcripts seen in cells microinjected with pTK or pAV-2/ΔTK-A. The structure and function of these four transcripts are not known; it is possible that the transcripts are promoted by cellular sequences flanking integrated plasmid DNA.

Analysis of *tk* RNA in Cells Transformed by Plasmids with a Complete *tk* Gene and RSV Enhancer

We extended our examination of HSV *tk* RNA in transformed cells to include cells that received plasmids containing a complete *tk* gene as well as the enhancer region of RSV DNA (Figure 2, lanes D–J). A 1.4 kb HSV *tk* RNA, presumably mRNA transcribed from the native HSV *tk* promoter (see lanes A and I), is common to all these cell lines. Moreover, the abundance of HSV *tk* mRNA (1.4 kb) is similar to that of the presumptive HSV *tk* mRNA in all TK^+ transformants, regardless of whether the HSV *tk* gene was accompanied by an RSV enhancer or whether transcription was promoted by HSV or RSV sequences. This conclusion was sustained by examination of RNA from a total of 29 transformed cell lines: 2 lines transformed by pTK, 6 lines transformed by pAV-2/ΔTK-A, 2 lines transformed by pAV-2/ΔTK-B, 12 lines transformed by pAV-1/TK-A, and 7 lines transformed by pAV-1/TK-B. Figure 2 (lanes H, I, and J) shows RNA from three cell lines analyzed

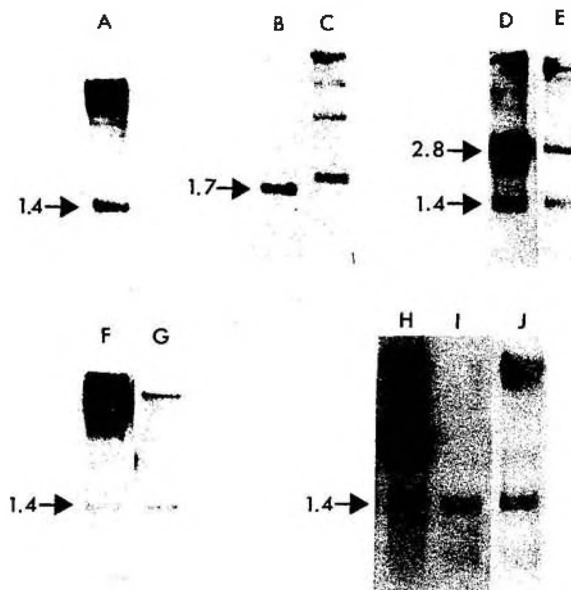

Figure 2. Transcription of the HSV *tk* Gene in TK^+ Colonies Can Be Promoted by Either the RSV LTR or HSV *tk* Promoters

Poly $(A)^+$ RNA (5 μg) from TK transformants was electrophoresed in formaldehyde–agarose gels, transferred to nitrocellulose membranes, and hybridized with an HSV *tk* probe as described in Experimental Procedures. Arrows denote positions of RNAs of designated lengths, as deduced from RNAs of known size. (Lanes A and I) RNA from cell line microinjected with pTK DNA. (Lane B) RNA from a cell line microinjected with pAV-2/ΔTK-A. (Lane C) RNA from a cell line microinjected with pAV-2/ΔTK-B. (Lanes D, E, and H) RNA from cell lines microinjected with pAV-1/TK-A. (Lanes F, G, and J) RNA from cell lines microinjected with pAV-1/TK-B.

on the same agarose gel to provide a direct comparison of the amount of the 1.4 kb HSV *tk* mRNA. Presumably, the enhancer sequence augments the chances that a level of TK necessary for biochemical transformation will be produced in any microinjected cell. The mechanism likely to be responsible for this effect will be considered below.

Both the RSV and the HSV *tk* promoters appear to be active in cells transformed by plasmids containing a complete *tk* gene and an RSV enhancer fragment. In addition to the native 1.4 kb HSV *tk* transcript, transcripts of 2.8–3.1 kb were present in lines transformed with pAV-1/TK-A. These transcripts annealed with both HSV *tk* probe (Figure 2, lanes D, E, and H) and RSV U5 probe (data not shown). The RSV U5 probe revealed discrete species of 3.1 and 2.8 kb, but only the 3.1 kb RNA species annealed with RSV U3 probe (data not shown), implying that the transcripts were promoted by both of the tandem LTRs in pAV-1/TK-A and polyadenylated at the HSV *tk* polyadenylation site (see Figure 3 for structure of this plasmid). The amount of these hybrid transcripts varied greatly among the transformed cell lines; for example, cell line Sc-6 (Figure 2, lane D) has 10 to 20 times more 2.8–3.1 kb RNA than does cell line Sc-4 (Figure 2, lane E). Hybrid transcripts of 2.8–3.1 kb were not observed in cell lines transformed by plasmids containing the complete HSV *tk* gene and the RSV enhancer DNA in opposite transcriptional orientations

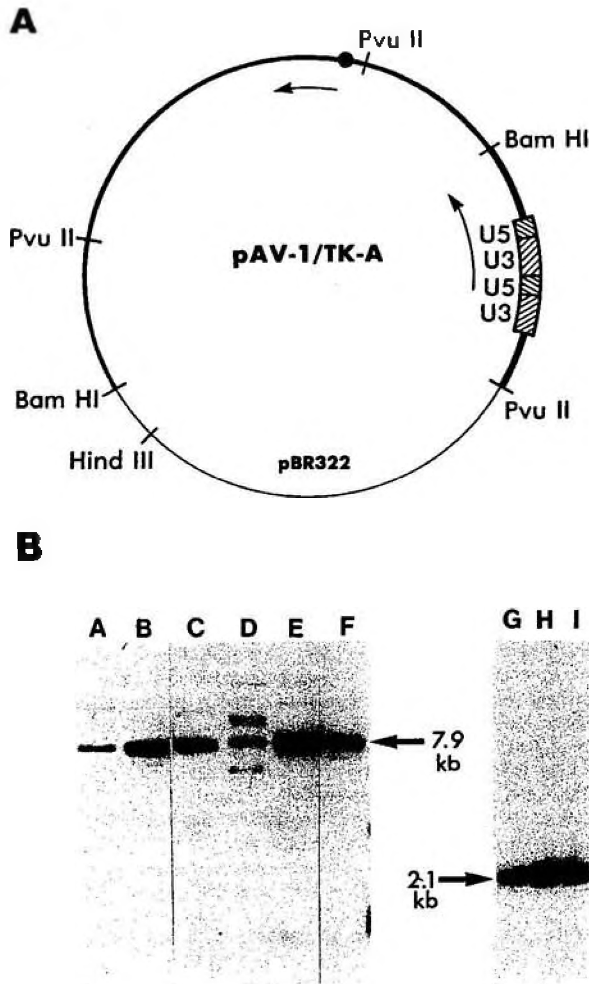

Figure 3. Structure of Microinjected DNA in TK⁺-Transformed Mouse L Cells

(A) Map of pAV-1/TK-A (7.9 kb), containing two RSV LTR sequences.

(B) The form of pAV-1/TK-A DNA in TK⁺-transformed cell lines was analyzed by restriction endonuclease digestion of high molecular weight cell DNA with Hind III (lanes A–C), with BglII (lanes D–F), and with Pvu II (lanes G–I). Cell line L2 (lanes A, D, and G) was made by microinjecting the linear form of pAV-1/TK-A produced by digestion with Hind III. Cell lines Sc-4 (lanes B, E, and H) and Sc-7 (lanes C, F, and I) were made by microinjecting supercoiled pAV-1/TK-A. Restriction digests were electrophoresed in 1% agarose gels, and DNA was transferred to nitrocellulose and annealed with either pAV-1/TK-A probe (lanes A–F) or RSV U5 probe (lanes G–H). The precise sizes of indicated fragments were determined from the mobility of known plasmid fragments.

(Figure 2, lanes F, G, and J). In these cells there is a variable amount of high molecular weight RNA that anneals with HSV *tk* probe. These large RNAs anneal to probes for all components of the microinjected plasmids (data not shown); they could in part be initiated within the RSV LTR and extended through the integrated tandem arrays of plasmid DNAs found in most cell lines (Figure 3B; Folger et al., 1982; unpublished results). However, high molecular weight species of RNA were also detected in some other cell lines, including lines transformed by pTK alone (Figure 2, lane A), and we have not rigorously defined their structure and function.

Table 3. Localization of the RSV Enhancer

"TATA" box (−30); Cap site (+1); (−229); (+101); U3; R-U5
Pvu II (−488); Nru I (−360); Sph I (−138); EcoRI (−51); BstE2 (+106)

Plasmid	Number of TK⁺ colonies per 10^3 TK⁻ cells injected: A	B
pAV-3/TK-JM	224	232
pAV-4/TK	248	254
pAV-3/TK-ΔN4	226	204
pAV-3/TK-ΔN5	30	18
pAV-3/TK-ΔN14	6	8
pAV-3/TK-ΔS8	10	12
pAV-11/TKΔE2	184	236
pAV-11/TKΔE1	198	NT

pAV-3/TK-JM contains a 439 bp RSV DNA fragment encompassing the 330 bp RSV LTR in a derivative of pBR322 with a single Bam HI site and a complete HSV *tk* gene. The two forms of pAV-4/TK were prepared by cloning both orientations of the 3.4 kb HSV *tk* Bam HI DNA into a derivative of pBR322 containing the region of RSV DNA from position −488 to −51 (Figure 1). The remaining plasmids were constructed by cloning the 3.4 kb HSV *tk* Bam HI DNA fragment in both orientations (A and B) into the Bam HI site of the RSV DNA deletion mutants; these were derived from a plasmid containing RSV DNA (positions −488 to +106, Figure 1) by Bal 31 nuclease digestion as described in Experimental Procedures. The coordinates of the deletions, determined by direct DNA sequencing are as follows: pAV-1/TKΔN4, −284 to −411; pAV-1/TKΔN5, −240 to −483; pAV-1/TKΔN14, −182 to position 2120 in pBR322; pAV-1/TKΔS8, −142 to −116; pAV-11/TKΔE2, −89 to +5, and pAV-/TKΔE1, −143 to +25.

Localization of the Enhancing Region in the RSV DNA

The plasmids described above (Table 1) contain the 330 bp RSV LTR flanked by 251 bp and 425 bp of RSV DNA (Figure 1). Several plasmids containing deletions within the RSV DNA were prepared in order to define the portions necessary for enhancement. The HSV *tk* gene was cloned into each of these derivatives in both orientations (A and B), and the resulting plasmids were tested for enhanced levels of TK transformation.

Table 3 presents the structure and the transformation efficiency of each of these plasmids. Removal of all sequences beyond the 3′ end of the LTR, the R-U5 region, and over 50 bp of U3 (including the TATA box and cap site) does not affect the enhancement phenomenon (pAV-4/TK in Table 3). Overlapping deletions that extend to 89 and 143 bp upstream from the cap site (pAV-11/TKΔE2 and pAV-11/TKΔE1) also fail to inhibit enhancement, suggesting the active sequence lies 5′ to this region. However, a smaller deletion (pAV-3/TKΔS8) that removes one base pair less than pAV-11/TKΔE1 in the 5′ direction lacks any enhancing activity, implying that the 5′ ends of the deletions in pAV-3/TKΔS8 and pAV-11/TKΔE1 are at or near the 3′ boundary of the region critical for enhancement. Presumably, the ΔE1 deletion does not eliminate features necessary for enhancement; those features must be ab-

sent from the sequence generated by the ΔS8 deletion, but their nature is not apparent from inspection of the sequences. Deletions extending toward the 5′ boundary of the LTR (pAV-3/TK-JM and pAV-3/TKΔN4) show that sequences 50 bp or more upstream from the LTR are expendable in the enhancement test. The 244 bp deletion in pAV-3/TKΔN5 that removes all but 10 bp upstream from the LTR markedly but incompletely reduces the enhancer effect; a deletion that extends 48 bp into the U3 region (pAV-3/TKΔN14) completely inactivates enhancement.

From this analysis we can tentatively assign the RSV enhancer function to an 88 bp sequence in the U3 region of the LTR, 142–229 bp upstream from the cap site, plus at least part of a 50 bp sequence that resides on the 5′ side of the LTR normally found at the 3′ end of proviral DNA in infected cells (see Discussion). Definition of the precise size and sequence of the RSV enhancing region will require the use of more deletion and substitution mutants that affect the implicated region, as well as smaller alterations within the sequence.

Localization of the Enhancing Region in SV40 DNA

In previous studies, a high level of TK^+ transformation was obtained when a 1000 bp fragment of SV40 DNA was cloned into a plasmid containing the HSV *tk* gene (Capecchi, 1980). A 72 bp repeat sequence, 80 bp upstream of the cap site for the early mRNA molecules, is important for expression of the SV40 early genes (Gruss et al., 1981; Benoist and Chambon, 1981; Gluzman et al., 1980; Fromm and Berg, 1982). This region is also present in the SV40 DNA fragment that enhanced TK transformation in the earlier studies (Capecchi, 1980). To define more precisely the SV40 sequences required for enhancement, we cloned a 162 bp SV40 DNA fragment (defined by an Nco I site and a Pvu II site; Figure 1), containing only one copy of the 72 bp repeat, into the Bam HI site of pTK-JM in both orientations (pTK/SV-A and pTK/SV-B). Enhanced transformation was observed with both of these plasmids (Table 4). Thus a region of SV40 DNA sufficient for enhancement of TK transformation is between 21 and 255 bp upstream from the TATA box for SV40 early gene mRNAs in wild-type DNA with two copies of the 72 bp repeat; this region lacks the origin of replication (Figure 1). No appreciable sequence homology was apparent when the known nucleotide sequences of the enhancing regions of RSV and SV40 DNA were compared (Swanstrom et al., 1981; Fiers et al., 1978).

To show directly that the origin of DNA replication was not an enhancing sequence, the HSV *tk* gene was cloned in both orientations into a plasmid containing a 316 bp region of SV40 DNA that contains the origin of SV40 DNA replication but lacks the 72 bp repeats, generating pSV-0/TK-A and pSV-0/TK-B. Microinjection experiments show that an intact origin of SV40 DNA replication is not sufficient for enhanced TK transformation (Table 4).

Table 4. Transformation Efficiencies Obtained by Microinjecting Plasmid DNA Containing the HSV *tk* Gene and SV40 DNA

Structure of Microinjected DNA	Number of TK^+ Colonies per 10^3 TK^- Cells Injected
pSV-0/TK-A (Sa RI RI Ba Bg Ba Sa)	15
pSV-0/TK-B (Sa RI RI Ba Ba Sa)	5
pTK/SV-A (H3 Bg/Ba Ba Pv H3)	255
pTK/SV-B (H3 Ba Bg/Ba Pv H3)	235

The heavy lines denote SV40, the medium lines HSV *tk*, and the light lines plasmid vector DNA. Each orientation of the 3.4 kb HSV *tk* Bam HI fragment was cloned in the Bam HI site of pSV-ori (a derivative of pBR322 containing the Eco RII fragment surrounding the origin; Figure 1B) to yield pSV-0/TK-A and pSV-0/TK-B. The open circle denotes the origin of SV40 DNA replication. Arrows denote the direction of HSV *tk* transcription. pTK-SV-A and pTK-SV-B contain a 173 bp DNA fragment with a single copy of the 72 bp repeat of SV40 DNA (open rectangle) in the Bam HI site of pTK-JM (Table 3). Symbols are as in Table 1 and as follows. RI: Eco RI. H3: Hind III. Pv: Pvu II.

Effect of the RSV Enhancing Sequence on Integration and Gene Expression

Retroviral sequences in HSV *tk* plasmids could enhance stable TK transformation by increasing the frequency of plasmid integration or by augmenting transcription of the HSV *tk* gene. We first determined the influence of an LTR upon the site(s) of integration within the microinjected plasmid DNA by mapping integrated DNA in TK^+ cell lines. Subsequently, the effect of an LTR upon the frequency of integration was evaluated. Cells were microinjected with HSV *tk* plasmids, with and without the RSV LTR, grown in nonselective medium, and assayed for integrated HSV *tk* DNA. The cells containing integrated HSV *tk* DNA were tested for gene expression by transfer to selective (HAT) medium.

Microinjected Plasmids Containing RSV LTRs Do Not Integrate Preferentially via LTR Sequences

We examined the pattern of integration in mouse L cells microinjected with plasmids containing the HSV *tk* gene and the RSV LTR to determine whether the LTR mediates integration into the mouse cell genome as it does during natural infection. Our results are illustrated here with three cell lines: L2 is a TK^+ transformant produced by microinjecting a linear form of pAV-1/TK-A, and Sc-4 and Sc-7 are transformants that received supercoiled pAV-1/TK-A (Figure 3A). When DNA from these cells was digested with Hind III or with Bgl II, restriction enzymes that cleave the 7.9 kb plasmid pAV-1/TK-A once (Figure 3A), probe to pAV-1/TK-A DNA revealed one common major fragment of 7.9 kb and minor fragments unique to each digest (Figure 3B, lanes A–F). The unit-length 7.9 kb DNA band

suggests that a tandem array of pAV-1/TK-A is present in each cell line, a conclusion supported by experiments with other restriction endonucleases (data not shown). The minor bands probably represent fragments in which plasmid DNA is joined to cellular DNA. The intensity of the 7.9 kb Hind III DNA fragments indicates that the tandem arrays contain about 2–5 units. Integrated tandem arrays were also observed in similar studies by Folger et al. (1982). No free HSV *tk* DNA was detected in undigested samples of high molecular weight cell DNA from cells microinjected with pTK or with pAV-1/TK-A (data not shown).

To focus specifically on the RSV LTR region, high molecular weight DNA from the TK$^+$ transformants was digested with Pvu II, which has sites 2.1 kb apart and flanking the RSV LTRs, in pAV-1/TK-A (Figure 3A). Analysis with RSV U5 probe revealed a single major band at 2.1 kb in each DNA sample (Figure 3B, lanes G–I). The LTRs must occur mainly at internal positions within the tandem array in each of these cell lines and rarely at or near the sites of joining of pAV-1/TK-A DNA to cellular DNA, since no junction fragments were observed in this analysis. Thus, in contrast to natural infection, the LTR does not provide a preferred site for integration when either linear or circular DNA is delivered by microinjection; this implies that enhancement is unlikely to be due to provision of a favored integration site. These conclusions have been confirmed by analysis of additional microinjected TK$^+$ transformants, including some with single, unit-length insertions (Folger et al., 1982; unpublished results of P. A. L.), and by analysis of cells transformed after calcium-phosphate-mediated transfection (P. Luciw et al., unpublished data).

Plasmids Containing the HSV tk Gene with or without the RSV Enhancing Sequence Integrate at Similar Frequencies

We compared the number of cells that contained integrated HSV *tk* DNA after the cells were microinjected with pTK DNA or with pAV-1/HSV-TK-A DNA (Figure 3A) and propagated in nonselective medium. The experimental strategy is outlined in Figure 4. A few cells (1–6) were seeded onto coverslips in individual tissue culture plates. The cells on each coverslip were microinjected, and cells from each coverslip propagated in nonselective growth

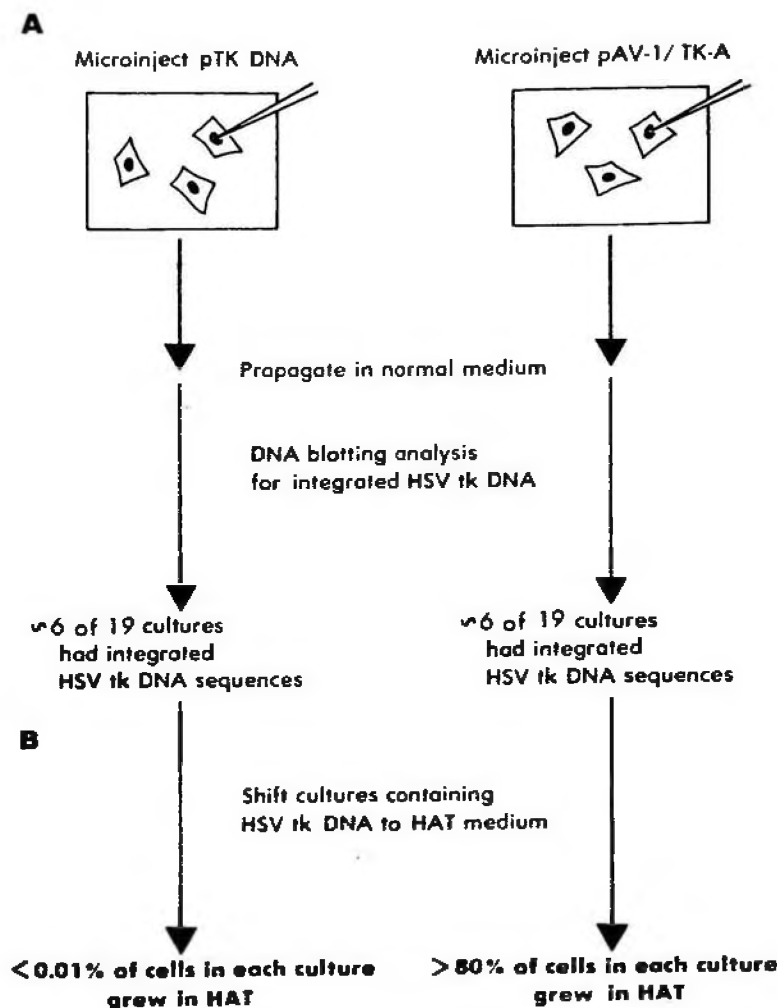

Figure 4. Analysis of Integration and Expression of the HSV *tk* Gene in Cells Maintained in Normal (Nonselective) Medium

(A) TK$^-$ mouse L cells were seeded on coverslips at a density of ~3 cells per coverslip in a 60 mm tissue culture dish. Each cell on a coverslip was microinjected with ~25 copies per cell of pTK or pAV-1/TK-A (both linearized with Hind III) and propagated in normal medium. After ~30 generations, high molecular weight cellular DNA was extracted from a portion of each culture, digested with Bam HI or Hind III, electrophoresed in agarose gels, and transferred to nitrocellulose membranes. ^{32}P-labeled pTK DNA probe was used to detect HSV *tk* sequences. The indicated numbers of cultures were found to contain integrated HSV *tk* DNA (as analyzed in Figure 5).

(B) Cells from each culture containing HSV *tk* DNA were seeded in 100 mm plastic tissue culture dishes, allowed to attach in normal medium, then shifted to HAT selective medium and replenished at intervals of 3–4 days with fresh HAT medium. After 2 weeks, the TK$^+$ colonies in each dish were counted (Table 5).

medium for approximately 30 generations. High molecular weight cell DNA from each culture was analyzed for the presence of HSV *tk* sequences after digestion with Bam HI, which cleaves twice in each plasmid, and with Hind III, which cleaves once in each plasmid. Figure 5 illustrates a portion of the analysis. HSV *tk* DNA was found in approximately 6 of 19 cultures receiving either plasmid. Digestion with Bam HI yielded a 3.4 kb DNA fragment containing HSV *tk*. A unit-length DNA species (7.8 or 7.9 kb) was produced by Hind III digestion in all of the cell lines containing HSV *tk* (Figure 5), indicating the presence of tandem arrays (see also Figure 3B and Folger et al., 1982). In general, the amount of DNA in cells receiving either of the two plasmids was similar to that in cells selected in HAT medium directly after microinjection (lanes I, I′, S, and S′). However, the amount of plasmid DNA in some cultures (see lanes H, H′, N, and N′) was relatively low, suggesting that only a portion of the cells maintained plasmid DNA. (Faint bands in lanes F and F′ are not visible in the photographs of the autoradiograms.) A rigorous determination of the number of copies per cell and their organization will require that the cells from each culture be cloned and subsequently analyzed for integrated HSV *tk* DNA. Nevertheless, the available results demonstrate that HSV *tk* plasmids with or without a retrovirus enhancing sequence integrate into cell DNA at similar frequencies. Thus the enhancement phenomenon seems unlikely to be attributable to an effect upon the efficiency of integration.

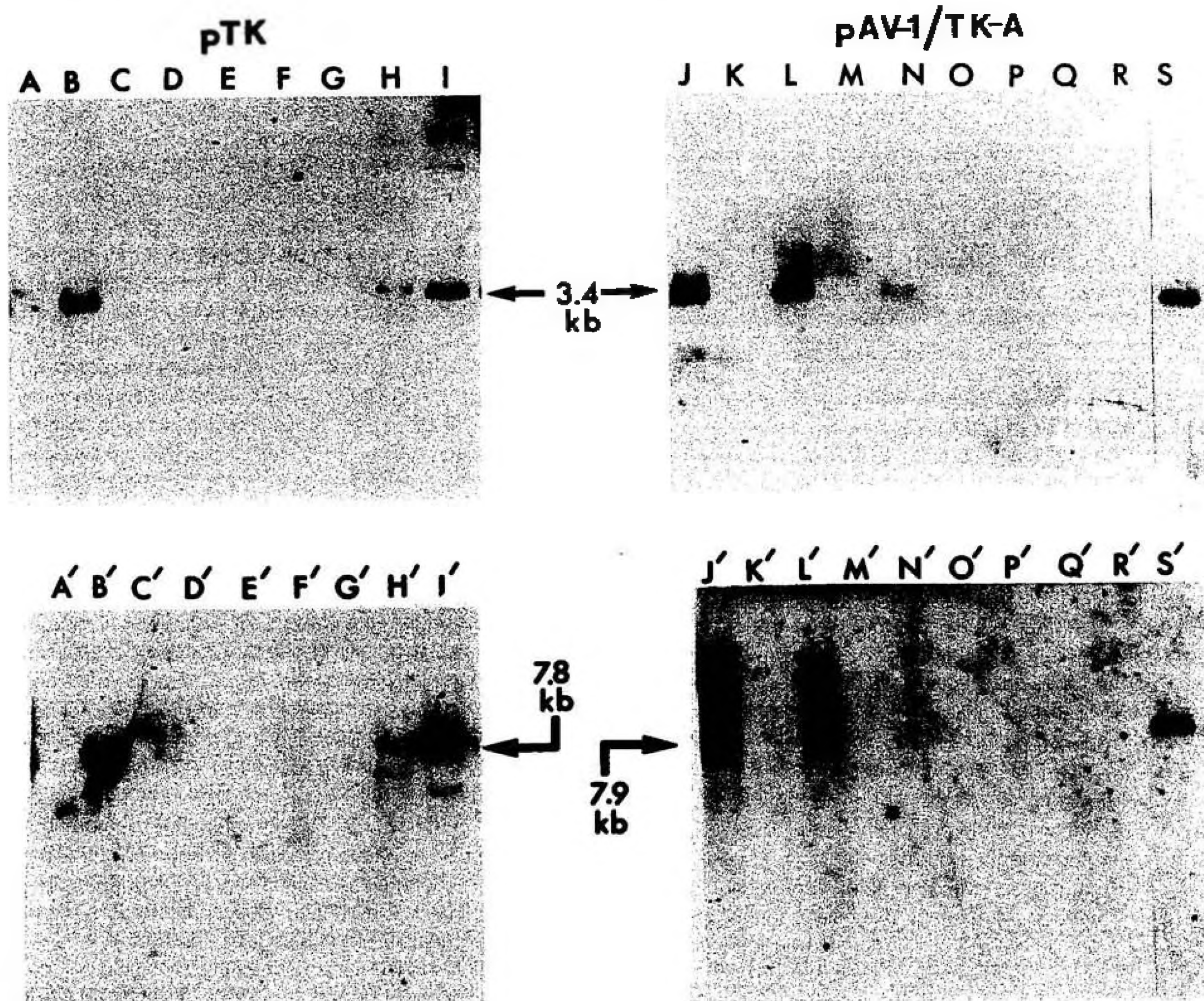

Figure 5. Analysis of HSV *tk* DNA in Cells Microinjected with Either pTK or pAV-1/TK-A DNA and Maintained in Nonselective Medium

TK^- mouse L cells on individual coverslips were microinjected with pTK or PAV-1/TK-A DNA (as detailed in Figure 4) and maintained in normal medium for approximately 30 cell generations. A portion of the cells propagated from each coverslip was used to prepare high molecular weight cellular DNA. This DNA was digested with Bam HI or with Hind III, electrophoresed in agarose gels, transferred to nitrocellulose membranes, and annealed with ^{32}P-labeled probe made with the 3.4 kb Bam HI DNA fragment containing HSV *tk*. (Lanes A–I) DNA from cells microinjected with pTK, digested with Bam HI. (Lanes A′–I′) DNA from cells microinjected with pTK, digested with Hind III. (Lanes J–S) DNA from cells microinjected with pAV-1/TK-A, digested with Bam HI. (Lanes J′–S′) DNA from cells microinjected with pAV-1/TK-A, digested with Hind III. (Lanes I and I′; S and S′) Markers obtained by digesting DNA from cell lines microinjected respectively with pTK or pAV-1/TK-A and selected in HAT medium.

Table 5. Plating Efficiency of Nonselected Microinjected Cell Lines after Transfer to Selective Medium

Cell Line	Microinjected Plasmid	HAT-Resistant Colonies/10^6 Cells Plated
44	pTK	150
46	pTK	5
53	pTK	120
74	pTK	110
80	pTK	1
82	pTK	20
1	pAV-1/TK-A	$\sim 10^6$
5	pAV-1/TK-A	$\sim 10^6$
15	pAV-1/TK-A	$\sim 10^6$
24	pAV-1/TK-A	$\sim 10^6$
31	pAV-1/TK-A	$\sim 10^6$
33	pAV-1/TK-A	$\sim 10^6$

TK^- L cells were microinjected with pTK or with pAV-1/TK-A and propagated in nonselective medium as described in the text and outlined in Figure 4. The resulting cell lines shown to contain plasmid DNA (see Figure 5) were tested for expression of HSV *tk* by plating in HAT medium at 10^3, 10^4, 10^5, and 10^6 cells per 100 mm dish. Colonies were counted after approximately 10 days, and results are expressed as number of HAT-resistant colonies per 10^6 cells. DNAs from some of these lines were analyzed in the experiment shown in Figure 5 (lane B, cell line 74; lane F, cell line 80; lane H, cell line 82; lane J, cell line 24; lane L, cell line 31; lane N, cell line 33).

The RSV Enhancing Region Activates Expression of Integrated HSV *tk* DNA

The cells from cultures harboring pTK DNA or pAV-1/TK-A DNA were used to examine the effect of the RSV enhancing sequence on expression of integrated HSV *tk* DNA. Cells from each culture demonstrated to contain HSV *tk* DNA were shifted to selective (HAT) medium, and the surviving colonies were counted (Figure 4). A very small proportion (10^{-4} to 10^{-6}) of cells from each of the six lines containing integrated pTK DNA grew in HAT medium (Table 5; see Discussion). In marked contrast, more than 80% of the cells from each culture harboring pAV-1/TK-A DNA thrived in HAT medium (Table 5). We conclude that the retrovirus enhancing sequence increases TK transformation by facilitating expression of the HSV *tk* gene.

Discussion

Identification of Enhancer Sequences

The work described here was initiated to investigate the integration and expression of retroviral DNA after microinjection into cultured cells. Early in these studies we unexpectedly found that portions of RSV DNA in recombinant plasmids containing the complete HSV *tk* gene markedly enhanced the frequency of transformation of TK^- L cells to a TK^+ phenotype, regardless of the position or orientation of the RSV DNA relative to HSV *tk*. This phenomenon appears similar to a situation we have observed in ALV-induced B-cell lymphomas, in which high levels of expression of the cellular oncogene, *c-myc*, are associated with various arrangements of proviral insertions adjacent to the oncogene (Payne et al., 1982). In addition, it resembles the effect of SV40 sequences upon transformation by microinjected HSV *tk* DNA (Capecchi, 1980) and a number of other intriguing phenomena referred to as gene enhancement or activation (see below). We have therefore localized the sequences in RSV and SV40 DNA responsible for enhanced transformation and examined the mechanism of enhancement in this system.

The regions of RSV and SV40 DNA necessary for enhancement of TK transformation are small (less than 155 bp and 162 bp, respectively) and positioned similarly within the RSV and SV40 segments of our plasmids (i.e., about 100–250 bp upstream from initiation sites for transcription). Very little homology is apparent when these RSV and SV40 sequences are compared. A short sequence (GTGGAA), at −124 to −129 in the RSV LTR, is homologous to the core enhancer element found in SV40 (Weiher et al., 1983). Another 5 bp sequence, TGGTAA, at positions −185 to −190 in the RSV LTR, is consistent with the consensus sequence proposed by Weiher et al. (1983). However, these homologies may be coincidental; experiments that explicitly test the role of these sequences in the enhancement phenomenon are now required.

The sequence required for maximal enhancement by RSV DNA in the assay used here is normally present only at the 3′ end of naturally acquired proviruses, since it appears to include 10–50 bp of viral sequence flanking the 5′ boundary of the LTR from circular DNA, as well as about 88 bp from U3 (Table 3). We note that the evidence for including sequence outside the LTR in the enhancer region depends upon results with a single deletion mutant (pAV-3/TKΔN5); it is, of course, possible that the mutation created a sequence inhibitory to enhancement. Assuming the domain extends beyond the LTR, how can the enhancer influence viral gene expression during natural infection? Since the enhancer affects HSV *tk* expression in *cis* regardless of position, perhaps the enhancer unit at the 3′ end of proviral DNA facilitates efficient expression from the 5′ LTR. Alternatively, sequences within the LTR may suffice for efficient expression from the RSV promoter itself. More experiments will be required to settle this issue. Nevertheless, it is notable that all of the ALV-induced tumors in which the LTR is improperly positioned for use as a promoter for *c-myc* retain the 3′ LTR and at least a few hundred base pairs of adjacent viral sequence (Payne et al., 1981, 1982).

We have found RSV DNA to contain an efficient promoter as well as an enhancer. High levels of TK transformation are obtained when the RSV LTR functions directly to promote transcription of the HSV *tk* gene (Table 2). Moreover, the RSV LTR initiates transcription under conditions in which expression of the HSV *tk* gene can proceed from its native promoter under the influence of the RSV enhancer (Figure 2). Conclusions about the effectiveness

of the RSV LTR as a promoter in mammalian cells must, however, take into account previous observations about viral gene expression in rodent cells infected with RSV. It appears that many rodent cells acquire an intact provirus after infection with RSV, although only a few are transformed (Boettiger, 1974). These results need to be reconciled with the finding that the RSV LTR is generally an effective promoter in similar cells in our experiments. One possibility is that the level of expression of the viral transforming gene (*v-src*) required for morphological transformation of a rat fibroblast is much higher than the level of HSV *tk* expression required for biochemical transformation of a TK^- mouse L cell. Another possibility is that the manner in which the DNA is delivered (e.g., by infection or microinjection) affects the frequency of expression. Both possibilities suggest that the integration site may influence expression from retroviral DNA. We observed that the amount of polyadenylated RNA apparently promoted by the RSV LTR (Figure 2, lanes D and E) can vary greatly in different TK-transformed cell lines; this may also be a consequence of an effect of integration site on expression of RSV DNA.

Mechanisms of Enhancement

Phenomena similar to the enhancement of TK transformation we have described have been assigned to regulatory elements of the genomes of SV40 virus (Moreau et al., 1981; M. Fromm and P. Berg, personal communication), polyoma virus (deVilliers and Schaffner, 1981; Tyndall et al., 1981), bovine papilloma virus (Lusky et al., 1983), and Moloney sarcoma virus (Levinson et al., 1982; Jolly et al., 1983; Kriegler and Botchan, 1983). Portions of at least two of these genomes (SV40 and MSV DNA) produce enhancement in our assay (Table 4 and unpublished data). However, we have identified viral regulatory regions that do not carry enhancers (e.g., a fragment of the LTR of RAV-0, an endogenous retrovirus of chickens; unpublished data), and enhancement is not limited to viral genomes. Enhancing activity has recently been found in a cellular sequence immediately upstream from the hamster adenosine phosphoribosyl transferase gene (M. R. C. and P. A. L., unpublished results) and in a primate cellular sequence homologous to a region near the SV40 origin of replication (Conrad and Botchan, 1982).

We considered two distinct mechanisms to explain enhancement of HSV TK transformation in our assays. RSV sequences encompassing the LTR in a plasmid containing the HSV *tk* gene could increase the efficiency of integration, the efficiency of *tk* gene expression, or both. Analysis of integrated DNA in TK-transformed mouse L cell lines revealed that the RSV LTR sequence is not the site of integration into host cell DNA (Figure 3B). Moreover, HSV *tk* DNA was found integrated in the same proportion of cells microinjected with either a plasmid containing the HSV *tk* gene or a plasmid containing both the HSV *tk* gene and RSV DNA sequences (Figures 4 and 5). Thus we can eliminate a direct involvement of enhancing sequences in integration or stabilization of DNA microinjected into mammalian cells.

Expression of the HSV *tk* gene was observed in a high proportion of cells derived from recipients of microinjected plasmids that contained the HSV *tk* gene and RSV DNA sequences; in contrast, only a very small proportion of cells derived from recipients of pTK DNA were able to grow in HAT medium (Figure 4). HSV *tk* gene expression may be dependent on the transcriptional activity of the site(s) of integration in the recipient cell genome; thus plasmids bearing HSV *tk* with an enhancer sequence may not be expressed at a level compatible with survival in HAT medium except in those infrequent cells in which a plasmid has integrated near an endogenous cellular enhancer. TK^+ transformants that arise at a low frequency in cells grown nonselectively after introduction of pTK DNA may result from secondary mutations (e.g., rearrangements) or from epigenetic phenomena (e.g., changes in chromatin structure or methylation status near the integration site). Further study of these transformants is required to distinguish among such possibilities.

Two distinct mechanisms are proposed for the enhancement phenomenon described here that take into account the fact that only certain regions of eucaryotic cell chromatin are transcribed. The enhancing regions could directly activate HSV *tk* transcription in many, but perhaps not all, sites of integration. Alternatively, the enhancing regions may direct microinjected DNA to integrate into a site of host chromatin that is transcriptionally active. These two possibilities are not mutually exclusive. To distinguish between these two mechanisms for activating HSV *tk* transcription, it will be helpful to examine the transcriptional or enhancer activity of the cellular integration sites in several independent TK^+-transformed cell lines.

Activation of transcription by enhancer sequences could be brought about by effects upon chromatin structure, DNA conformation, or affinities for factors required for transcription. Several related possibilities that may provide the mechanistic basis for enhancement have been discussed by other workers (Banerji et al., 1981; Benoist and Chambon, 1981 deVilliers and Schaffner, 1981). Discussions of mechanism, however, are complicated by the variety of criteria used to measure the effects of different enhancers upon different promoters in different types of cells. It is not known, for example, whether sequences that affect the strength of their native cellular and viral promoters when positioned upstream from the TATA box (Grosschedl and Birnsteil, 1980; Benoist and Chambon, 1981; Tsuda and Suzuki, 1981; McKnight et al., 1981; Gruss et al., 1981) are structurally and functionally equivalent to sequences that enhance the activity of heterologous promoters regardless of orientation and position (Banerji et al., 1981; deVilliers and Schaffner, 1981; Moreau et al., 1981; M. Fromm and P. Berg, personal communication; Levinson et al., 1982; Payne et al., 1982; Jolly et al., 1983; Kriegler and Botchan, 1983; this work). Furthermore, the effects of enhancers on heterologous promoters

has been assessed by a variety of tests for gene expression shortly after DNA transfection (Banerji et al., 1981; Moreau et al., 1981), during virus replication (Levinson et al., 1981), or after stable transformation by DNA introduced by transfection (Conrad and Botchan, 1982) or by microinjection (Capecchi, 1980; this work). We have obtained provisional evidence that enhancer-promoter combinations functional in one set of experimental conditions may not work in another. When we introduced recombinant plasmids containing the RSV enhancer region and the HSV *tk* gene into mouse L cells by calcium-phosphate-mediated DNA transfection and measured RNA synthesized from the HSV *tk* promoter 48 hr later, we found no effect of the RSV DNA upon the abundance of the *tk* RNA (unpublished data of P. A. L.). This may mean that integration of plasmid DNA into the host chromosome is required to elicit the enhancing effect that we have observed upon stable transformation of cells after microinjection. Another complication arises from the studies of Botchan and Khoury and their coworkers (personal communications), who have observed that the species of origin of the host cell may appreciably influence the activity of certain enhancer sequences.

Experimental Procedures

Cell Culture and Microinjection of Nuclei with Micropipettes

The culturing of TK^- mouse L cells and selection of TK^+ transformants has been described elsewhere (Wigler et al., 1977). The method and equipment for injecting DNA directly into the nuclei of viable mammalian cells in monolayer culture has been detailed by Capecchi (1980).

Approximately five to ten cells were seeded per 0.5 cm × 0.5 cm glass coverslip and maintained in growth medium in a 60 mm plastic petri dish for about four cell doublings. DNA was injected into 5–20 cells per coverslip. The small number of cells per dish receiving an injection dictates that many of the dishes in each experiment contain no transformed colonies, or very few. After injection, cells were incubated for 24 hr in nonselective medium at 37°C in a 5% CO_2 incubator and then switched to HAT medium and observed periodically over 3 weeks for the presence of large healthy colonies. Initially, several small TK^+ colonies are observed since nearly all of the cells receiving an injection express TK activity (Capecchi, 1980; Folger et al., 1982). During the first 2 weeks following injection, many of these colonies stop growing. The large surviving colonies presumably are composed of cells in which the injected DNA has integrated into the host cell genome in a transcriptionally competent state.

Cloning Procedures and Plasmid Preparation

The E. coli strain HB101 was used to clone and propagate the plasmids described in this study. Plasmid cloning procedures, including restriction endonuclease digestions, alkaline phosphatase treatments, ligation, bacterial transformation, screening recombinant plasmids, and agarose gel electrophoresis of DNA, are described by Maniatis, Fritsch, and Sambrook (1982). Restriction endonucleases were obtained from New England BioLabs and Bethesda Research Labs; T4 DNA ligase was from New England Biolabs; alkaline phosphatase (calf intestinal) was obtained from Boehringer Mannheim. Collaborative Research was the source of octameric deoxynucleotide linkers containing specific restriction endonuclease sites. For preparative purposes, large cultures of bacteria harboring plasmids were grown, and bacteria were harvested and treated with lysozyme and Triton X-100 and used for purification of plasmid DNA. Bacterial RNA was removed by passing plasmid DNA preparations through a BioRad A50 gel filtration column according to DeLorbe et al. (1980). The supercoiled form of plasmid DNA was isolated after buoyant density centrifugation in cesium chloride gradients.

Construction of Recombinant Plasmids

The structures of the recombinant plasmids used in this work are illustrated in Tables 1 to 4 and in Figure 3. A detailed account of the construction of these plasmids is available upon request.

Deletion Mutants Generated by Bal 31 Nuclease

pAV-3 DNA was digested with either Nru I or Sph I (Figure 1), treated with nuclease Bal 31 (1 U/μg DNA) for 1 min at 23°C in a buffer containing 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.5 M NaCl. The enzyme was removed by phenol extraction. After ethanol precipitation, the linear DNA was recircularized with T4 DNA ligase, and deletion derivatives were recovered by transforming HB101. Three deletion derivatives at the Nru I site (pAV-3/TKΔN4, pAV-3/TKΔN5, and pAV-3/TKΔN14) and one deletion derivative at the Sph I stie (pAV-3/TKΔS8) were prepared. Similarly, pAV-11 DNA was digested with Eco RI and then treated with nuclease Bal 31 to generate deletion derivatives. Two deletion mutants (pAV-11/TKΔE1 and pAV-11/TKΔE2) were isolated. pAV-3/TKΔN4, pAV-3/TKΔS8, and pAV-11/TKΔE1 were sequenced by Margarita Quiroga (Chiron Corp., Emeryville, CA.) by the method of Maxam and Gilbert (1980). Using the M13 cloning method with the dideoxy sequencing technique (Messing et al., 1981), Rick Najarian (Chiron Corp.) sequenced pAV-11/TKΔN5, pAV-11/TKΔN14, paV-3/TKΔS8, and pAV-11/TKΔE2. The extent of the deletion in RSV DNA for each plasmid is indicated in the legend to Table 3.

RNA and DNA Isolation and Blotting with ^{32}P-Labeled Probes

The oligo (dT)-cellulose batch method was used to prepare poly$(A)^+$ mRNA from mouse L cells as described by Varmus et al. (1981). mRNA was electrophoresed in an agarose gel system containing formaldehyde (Nusse and Varmus, 1982) and subsequently transferred to nitrocellulose membranes (Thomas, 1980). ^{32}P-labeled DNA probes to the 3.4 kb HSV *tk* Bam HI DNA fragment and to pBR322 DNA were prepared by using reverse transcriptase (supplied by J. Beard) to copy denatured template DNA in the presence of calf thymus DNA primers. ^{32}P-labeled RSV-U5 DNA probe was made in the endogenous reaction with purified Pr-C virus as described by Shank et al. (1978). ^{32}P-labeled RSV-U3 DNA probe was made by purified reverse transcriptase using purified RSV RNA (Schmidt-Ruppin A strain) and oligo (dT) primers (Shank et al., 1978). Analysis of cellular DNA was performed according to published protocols (Hughes et al., 1978).

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