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Host-specificities of papillomavirus, Moloney murine sarcoma virus and simian virus 40 enhancer sequences

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The bovine papillomavirus (BPV-1), Moloney murine sarcoma virus (MoMuSV) and simian virus 40 (SV40) genomes have been shown to contain sequences termed 'enhancers' which activate the expression of linked genes. DNA fragments containing these three enhancers have been inserted into recombinant plasmids upstream from the herpes simplex virus thymidine kinase *(tk)* gene, and their effect on *tk* expression monitored. Two types of assay have been used. Firstly, the ability of recombinant plasmids to transform TK$^-$ recipient cells to a TK$^+$ phenotype was measured. Secondly, the amount of *tk*-specific RNA and TK enzyme activity transiently expressed after DNA transfection was determined. Both types of assay gave similar results. The enhancers increased *tk* gene expression by regulating the amount of full length *tk* mRNA present shortly after transfection independent of gene copy number. Furthermore, marked species specificity in the relative efficiencies of different enhancers was observed, including that of the BPV-1 enhancer for the first time. The MoMuSV enhancer showed preference for murine fibroblasts, while the papillomavirus enhancer showed a marked preference for bovine cells. In contrast, the SV40 enhancer gave the same relative increase in *tk* gene expression in the murine, rat, bovine and human cells tested.

Key words: enhancers/gene transfer/papillomavirus/Moloney murine sarcoma virus/simian virus 40

Introduction

Viruses are host-specific parasites that encode proteins capable of specific interaction with cellular components. Species specificity has long been recognized in the early steps of viral infection such as adsorption, penetration and uncoating, but is less well understood in the later steps, i.e., transcription, replication and maturation of viral genomes which also involve interactions with host functions. The availability of cloned viral genomes and the establishment of efficient transfection techniques have made it feasible to circumvent the early steps of viral infection and examine subsequent interplays between viral nucleotide sequences and host functions.

A new class of viral regulatory elements, termed enhancers, has been recognized recently. A number of viral genomes including SV40 (Capecchi, 1980; Benoist and Chambon, 1981; Gruss *et al.*, 1981; Banerji *et al.*, 1981), polyoma virus (De Villiers and Schaffner, 1981; Tyndall *et al.*, 1981), bovine papillomavirus (BPV-1) (Campo *et al.*, 1983) and certain retroviruses (Blair *et al.*, 1980; Chang *et al.*, 1980; Huang *et al.*, 1981; Levinson *et al.*, 1982) harbour these enhancer elements in their genomes. It has been found that there is little if any sequence homology among these various elements (Laimins *et al.*, 1982; Weiher *et al.*, 1983). Enhancers are short sequences which can stimulate, by up to two orders of magnitude, the transcription of coding sequences from their own or other promoters (Banerji *et al.*, 1981; De Villiers and Schaffner, 1981; Wasylyk *et al.*, 1983). They act independently of orientation and at a distance with respect to the coding sequences (Banerji *et al.*, 1981; Moreau *et al.*, 1981; Wasylyk *et al.*, 1983; J.Lang *et al.*, in preparation). These properties clearly differentiate the enhancer sequence from the classical elements of a promoter (i.e., the TATA or CCAAT boxes) which map at a fixed distance from the 5′ end of the transcriptional unit.

We have used two sensitive and quantitative assays of gene activity to determine the effects of viral enhancer sequences on thymidine kinase *(tk)* gene expression. The results suggest that host specificity is one further property of viral enhancer elements.

Results

Long term transformation assays

The recombinants used in this study are shown in Figure 1. The SV40 enhancer-promoter fragment and the Moloney murine sarcoma virus (MoMuSV) and BPV-1 enhancer fragments are inserted upstream of the herpes simplex virus 1 (HSV-1) *tk* gene in plasmid pTK1. The relative positions of 'enhancers' and 'promoters' are indicated.

The effects of BPV-1, MoMuSV and SV40 enhancers on the ability of recombinant plasmids to transform TK$^-$ cells to a TK$^+$ phenotype are shown in Figure 2. The murine, rat and human cell lines used are described in Materials and methods. The following observations can be made. All three types of viral enhancers showed increased transformation activities in all three types of cells, and host-specific differences in transformation frequencies among the viral enhancer sequences were found when compared with pTK1. The most dramatic difference was the relatively lower enhancement of transformation of human 143 TK$^-$ and rat 4 TK$^-$ cells by the MoMuSV as compared with the SV40 enhancer, while the relative enhancement by the BPV-1 enhancer was approximately the same for the three different recipient cell lines. Transformation frequencies of rat 4 TK$^-$ and human 143 TK$^-$ cell lines were one to two orders of magnitude lower than mouse LATK$^-$ cells with all recombinants tested. We presume this reflects the reduced long term capacity of these cells to stably acquire and express DNA after treatment with calcium phosphate co-precipitate (see Materials and methods).

Short term transfection assays

Measurements of TK enzyme levels in transfected cells. To examine whether these differences in enhancement and host-specificities were reflected in short term transfection assays (Banerji *et al.*, 1981) expression of the HSV-1 specific *tk* gene was measured 48 h after treatment with DNA-calcium phosphate co-precipitates. Since there was no requirement for

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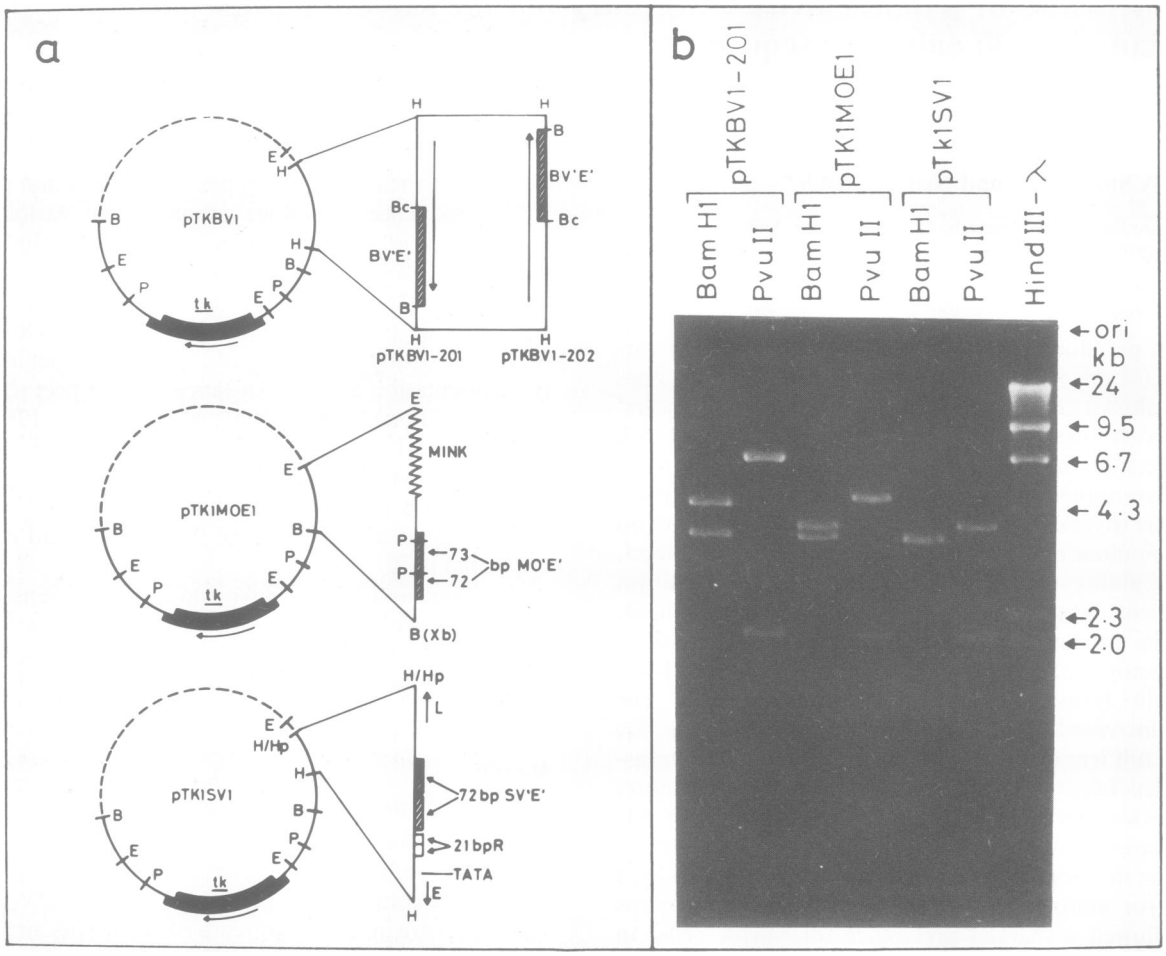

Fig. 1. (a) Recombinant plasmids carrying the viral enhancer sequences. pTKBV1-201 and -202 carry a 1175-bp *Hae*III fragment A of BPV-1 inserted in the 5′-3′ or 3′-5′ orientations using *Hind*III molecular linkers (Campo *et al.*, 1983). The BPV-1 enhancer-containing sequences has been localized within the *Bcl*I-*Bam*HI 0.6-kb DNA fragment (Chen *et al.*, 1982; M.Lusky, personal communication. pTKMOE1 carries a 530-bp *Eco*RI-*Xba*I fragment of cloned integrated MoMuSV carrying 230 bp of mink sequences upstream of the remaining 300 bp of viral LTR sequences (Dhar *et al.*, 1980). The *Xba*I site in MoMuSV LTR was converted to *Bam*HI using molecular linkers (Laimins *et al.*, in preparation). pTK1SV1 carries a 430-bp *Hpa*II-*Hind*III fragment of SV40 carrying the enhancer and the early and late promoters (Reddy *et al.*, 1978; Fiers *et al.*, 1978; Moreau *et al.*, 1981). The *Hpa*II site in SV40 DNA was fused to the *Hind*III site of pAT153 DNA. The broken lines indicate pAT153 sequences; the solid lines viral sequences; the outer heavy lines the structural *tk* gene; the inner heavy line the *tk* coding sequences; the hatched lines the viral sequences carrying the enhancer elements; the wavy line the mink sequences; TATA = TATA box, promoter homology sequences; the arrows indicate the direction of transcription. Enhancer regions are designated 'E'. The maps are not drawn to scale. E = *Eco*RI, B = *Bam*HI, H = *Hind*III, P = *Pvu*II, Bc = *Bcl*I, H/Hp = *Hind*III/*Hpa*II, B(Xb) = *Bam*HI (*Xba*I). **(b)** Restriction enzyme digests of recombinants shown in **(a)**. Bacteriophage λ DNA cut with *Hind*III was run in parallel as a marker.

TK$^-$ cells in this type of assay, cells from bovine conjuctiva (Moar *et al.*, 1981) at their 20th passage, designated 20 CON, were also included. The enzyme assay conditions were designed to suppress cell-coded TK enzyme and maximize the HSV-1 specific enzyme (Wilkie *et al.*, 1979). Furthermore, transfection with carrier DNA alone resulted in no increase in TK levels. As shown in Figure 3, the presence of each enhancer led to elevated levels of TK enzyme activity (compared with pTK1), in all of the cell lines tested. Moreover, a strong host-specific effect is readily seen. While the SV40 enhancer caused the same relative increase (compared with pTK1) in mouse, bovine and human cells, the MoMuSV enhancer had the greatest effect in mouse cells, and the BPV-1 enhancer the greatest effect in bovine cells.

To compare the SV40 result in human cells with the natural host (monkey cells), the transient expression assay was carried out in CV-1 monkey cells. In monkey cells, the SV40 enhancer caused only a 1.5 times greater increase in *tk* expression compared with human cells, confirming the relative lack of measurable host cell specificity.

Measurements of tk transcript levels in transfected cells. The relative levels of HSV *tk* transcripts in cells 48 h after transfection with the various DNAs were determined using an RNA spot hybridization assay (Spandidos *et al.*, 1981) and the results are shown in Figure 4 and Table I. Preliminary results showed that maximum levels of RNA were observed 48 h after transfection, and that the presence of enhancers did not alter the kinetics of appearance of *tk*-specific RNA (data not shown). All three enhancers caused increased levels of *tk* transcripts in mouse, rat, human and bovine cells. Once again, host-specificities for the enhancers were found and the pattern obtained was similar to that found with TK enzyme assays (Figure 3). The SV40 enhancer caused an ~20-fold increase (compared with pTK1) in the transient expression levels of *tk*-specific RNA in mouse, rat, human and bovine cells. In contrast, the MoMuSV enhancer caused an ~20-fold increase in murine cells, as compared with between a 4- and 8-fold increase in rat, bovine and human cells. Similarly, the

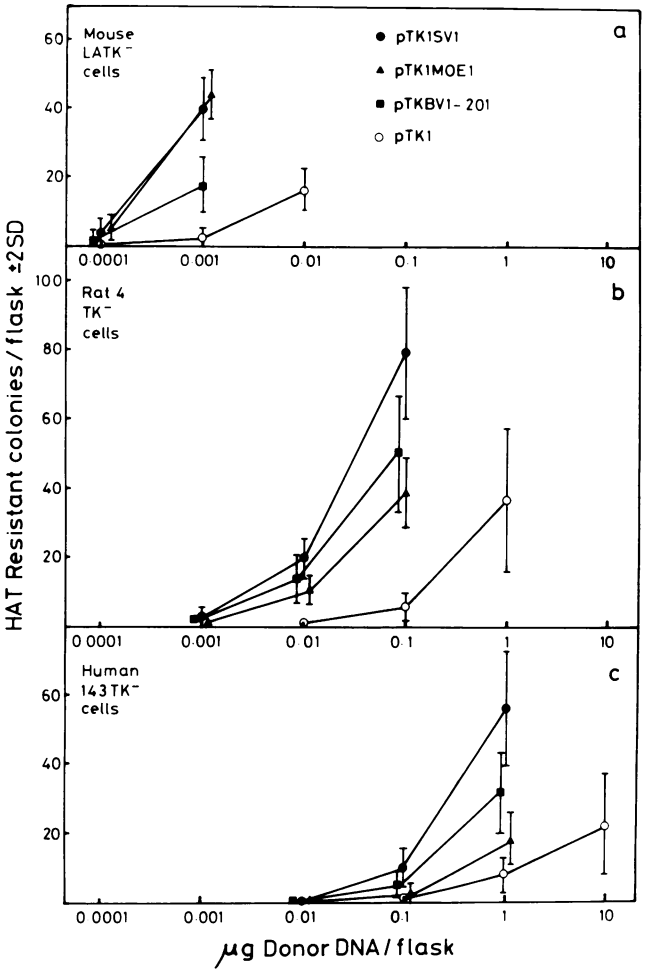

Fig. 2. Relationship between transformation frequencies and donor DNA concentration. Transformation of mouse LATK$^-$ **(a)** rat 4 TK$^-$ **(b)** and human 143 TK$^-$ **(c)** cells with pTK1 recombinant plasmids containing SV40, MoMuSV or BPV-1 enhancer sequences (see Figure 1) was performed as described in Materials and methods. The final DNA concentration at each point was 20 μg/ml during the precipitation step which was achieved by the addition of salmon sperm DNA as a carrier when 1 μg or less of recombinant DNA were used. Each point represents the average number of colonies of six independent measurements in three different experiments and the error bars represent ±2 SD. Transformation frequencies >100 colonies/flask obtained after addition of higher amounts of donor recombinant DNAs to recipient cells are not included in the figure since plateau levels were reached in these types of assays. ● pTK1SV1, ▲ pTK1MOE1, ■ pTKBV1-201 and ○ pTK1.

BPV-1 enhancer caused an ~20-fold increase in bovine cells compared with a 6- to 8-fold increase in mouse, rat and human cells. Although the RNA spot assay does not distinguish between authentic transcripts, i.e., transcripts initiated at the correct cap site(s), or transcripts initiated elsewhere in the donor DNA sequences, it is obviously a very sensitive assay and provides further support that enhancers act by altering transcriptional control.

The HSV-*tk* transcripts have been analyzed in a number of systems (Wilkie *et al.*, 1980; McKnight and Gavis, 1980; Roberts and Axel, 1982). Previous studies using Northern blot hybridization analysis had demonstrated the presence of two major RNA transcripts in stably transformed mouse cells: a predominant species of 1.3 kb and a second one of 0.9 kb (Roberts and Axel, 1982). As shown in Figure 5, in short term expression assays and in all three types of cells examined, *tk* transcript levels as detected by Northern blot hybridization were highly elevated in cells transfected with pTK1SV1, pTK1MOE1 or pTKBV1-201 as compared with cells transfected with pTK1 or salmon DNA controls. RNA transcripts of 1.3 kb and 0.9 kb were the predominant species, as was also found in LAT3, a stably transformed LATK$^+$ cell line with pTK1. A similar host-specific effect on transcript levels was also observed by this type of analysis and this has been confirmed by scanning across the 1.3-kb region (Figure 5b). Using pTKBV1-202 (Figure 1), in which the BPV enhancer fragment is in the opposite orientation, a similar host-specific effect in bovine conjuctival cells was observed (data not shown).

We have previously shown that at 48 h post-transfection of an SV40 recombinant in human cells, the donor DNA is assembled into regularly spaced nucleosomes and it is present in a high copy number per cell (Gilmour *et al.*, 1982). In the present study, the levels of pTKBV1-201 DNA at 48 h post-transfection have been also determined in mouse, rat, human and bovine cells using a DNA spot hybridization assay which employs whole cells (Spandidos *et al.*, 1981). It has been found that donor DNA levels were ~20 000–50 000 copies/cell, with the rat and human cells towards the lower values and the mouse and bovine cells towards the higher levels (data not shown). These results provide further support that the observed differences in transcript levels are the result of transcriptional control rather than differences in uptake or amplification of donor DNA, at least in short term transfections.

Discussion

The presence of enhancer sequences in the genomes of BPV-1 (Campo *et al.*, 1983), MoMuSV (Blair *et al.*, 1980; Levinson *et al.*, 1982) and SV40 (Capecchi, 1980; Benoist and Chambon, 1981; Gruss *et al.*, 1981; Banerji *et al.*, 1981, Wasylyk *et al.*, 1983) is well documented. Host specificities for the SV40 (Laimins *et al.*, 1982; De Villiers *et al.*, 1982; Byrne *et al.*, 1983) and MoMuSV (Laimins *et al.*, 1982) enhancer sequences have been reported recently. In the present study, using pTK1 as a positive internal control for each cell line tested, we measured the relative increase in *tk* gene expression obtained with the three enhancers tested. The results clearly show the preference of the MoMuSV enhancer for murine cells and the BPV-1 enhancer for bovine cells, but the SV40 enhancer gave approximately the same relative increase in *tk* gene expression with every cell line tested including CV-1 monkey cells (see Figures 2–5, Table I and text). This is the first demonstration of a host-specific effect for a papilloma virus enhancer. Our results with SV40 enhancer are contradictory to the recent claims that the SV40 enhancer also shows a host-specific effect (Laimins *et al.*, 1982; De Villiers *et al.*, 1982; Byrne *et al.*, 1983). However, most of these studies are flawed by the lack of internal controls to measure potential differences between different cell types in the ability to take up and express exogenously-supplied DNA. For example, in the present study the human 143 TK$^-$ cells expressed transient TK enzyme levels after transfection ~10 times lower than that of murine LATK$^-$ cells (see legend to Figure 3). The studies of Laimins *et al.* (1982) also claimed a host-cell effect for SV40 using activation of the chloramphenicol acetyl transferase (CAT) gene as a target. This study used the CAT gene under SV40 promoter control (lacking the enhancer) as an internal standard. However, the enzyme levels so obtained may have been too low to monitor

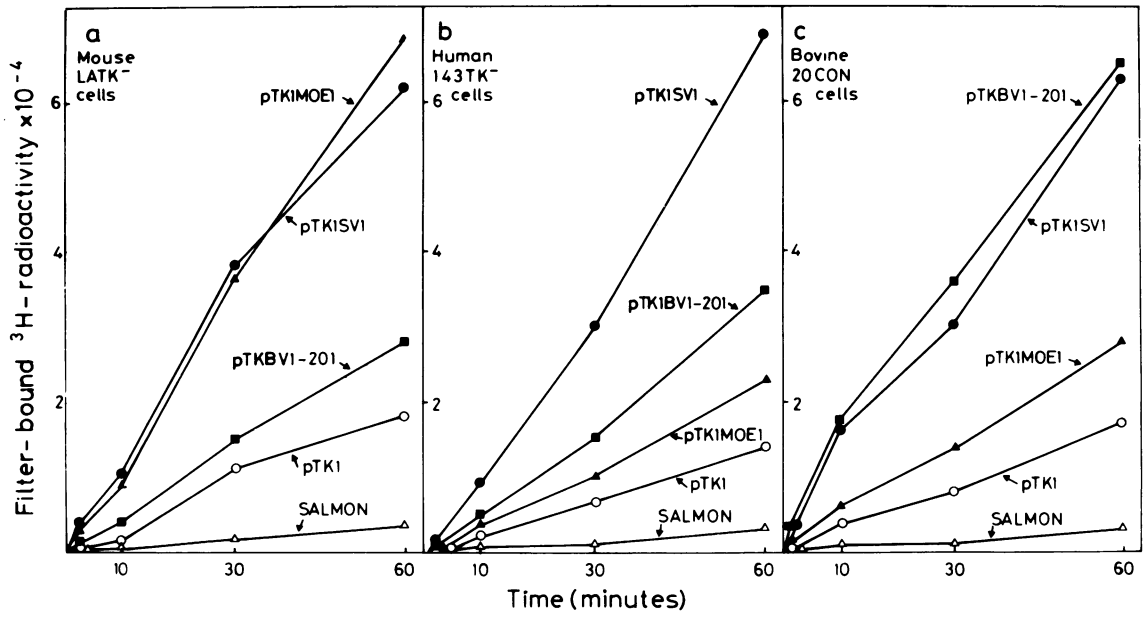

Fig. 3. Short term expression of HSV TK activity in mouse **(a)** human **(b)** and bovine **(c)** cells as determined by measuring conversion of [^{3}H]thymidine into thymidine monophosphate and higher phosphates. TK activity was determined in the presence of 0.2 mM TTP in extracts of 1 x 10^5 LATK$^-$, 1 x 10^6 143 TK$^-$ or 5 x 10^5 20 CON cells as described (Wilkie *et al.*, 1979). ● pTK1SV1, ▲ pTK1MOE1, ■ pTKBV1-201, ○ pTK1 and △ salmon DNA.

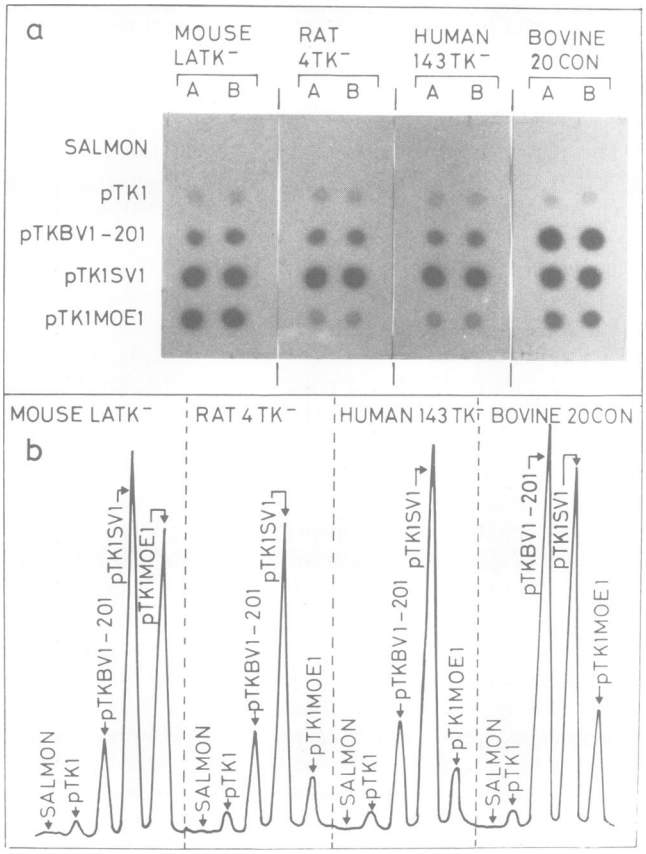

Fig. 4. RNA spot hybridization assays of HSV-*tk* transcripts at 48 h post-transfection of mouse, rat, human and bovine cells with salmon, pTK1, pTKBV1-201, pTK1SV1 and pTK1MOE1 DNAs. Total cell RNA from transfected cells was made as described (Spandidos and Paul, 1982). RNA spot hybridization assays have been described in detail previously (Spandidos *et al.*, 1981). 20 μg RNA was spotted in duplicates A and B and the filter was hybridized with ^{32}P-labelled 0.6-kb, *Bgl*II-*Sst*I, *tk* DNA probe by nick-translation (Rigby *et al.*, 1977). **(a)** The autoradiograph, and **(b)** scanning across A.

Table I. Short term expression of HSV *tk*-specific RNA in mouse, rat, human and bovine cells transfected with HSV *tk* recombinants[a]

Donor DNA	*tk*-specific RNA[b]			
	Mouse LATK$^-$	Rat 4 TK$^-$	Human 143 TK$^-$	Bovine 20 CON
Salmon	–	–	–	–
pTK1	1	1	1	1
pTKBV1-201	7	6	8	22
pTK1SV1	20	18	22	20
pTK1MOE1	19	5	4	8

[a]The experimental details are described in the legend of Figure 4.
[b]The autoradiographs (Figure 4a) were scanned (Figure 4b) and the averages of duplicates A and B for each RNA sample are given relative to pTK1.

adequately cell differences in DNA uptake and expression. The reasons for the differences in the present results and those of others (who also differ among themselves) is not yet clear. Another possibility is that the use of different target genes to measure enhancer effects may yield different results for as yet unknown reasons. It should be noted that the short term HSV-TK assay compares favourably with the CAT assay (Laimins *et al.*, 1982) and the previously used T-antigen expression assays (Laimins *et al.*, 1982; De Villiers *et al.*, 1983) in terms of sensitivity, speed and cost.

Our results also unambiguously show that the enhancers mediate gene expression by regulating the amount of full length *tk* mRNA present in transfected cells. This effect is unrelated to the DNA copy number present in the cells, although we cannot rule out local effects in the cells within the culture which are expressing TK activity. We favour the hypothesis that the enhancers affect the rate of primary transcription, although other effects such as stability of message or rates of RNA processing cannot be excluded. Furthermore, our finding that host-cell specificities can clearly be observed using the MoMuSV 'enhancer' alone (the 'promoter' region

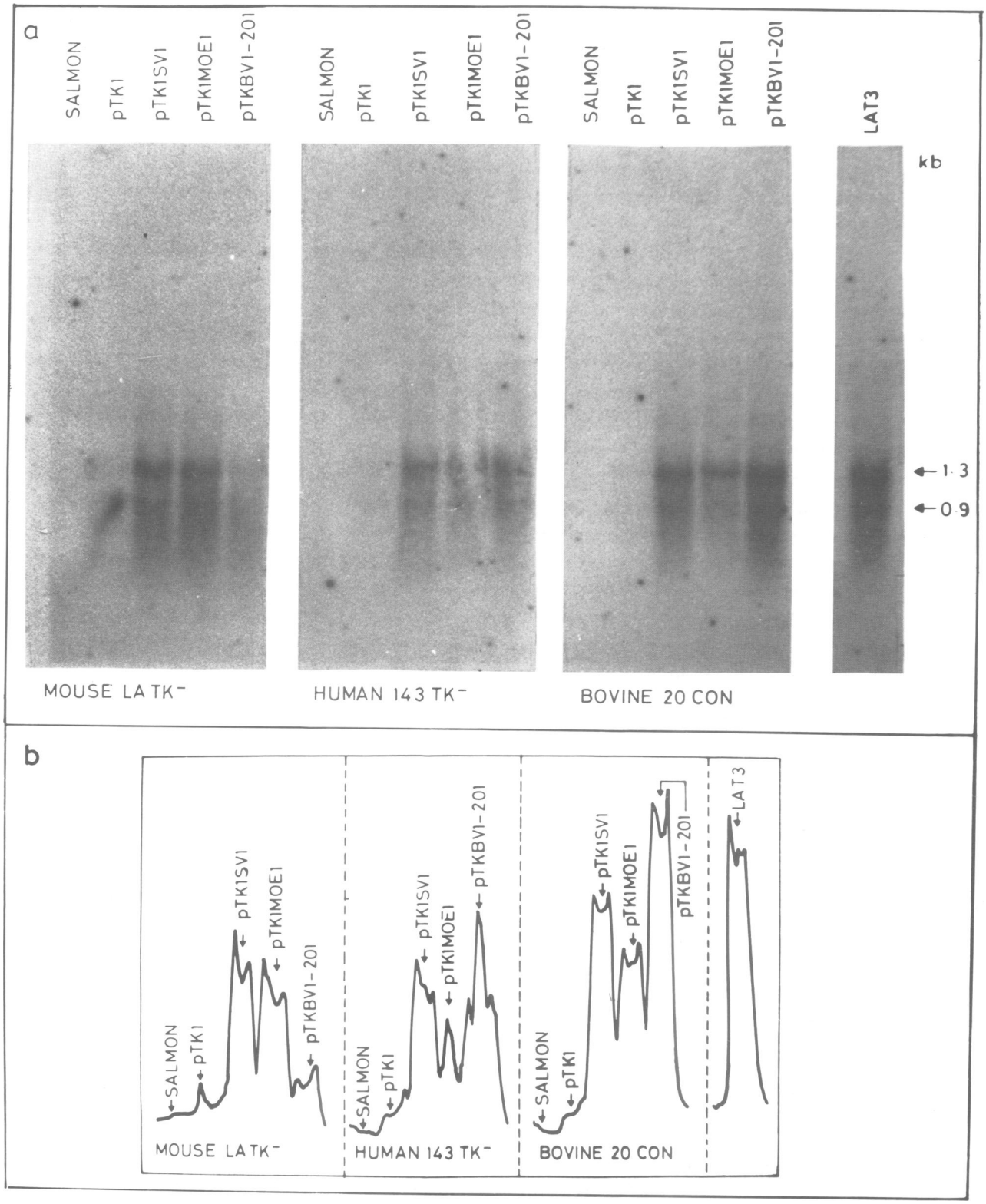

Fig. 5. *tk* RNA in transfected cells. 20 μg of total cell RNAs from mouse LATK⁻, human 143 TK⁻ or bovine 20 CON cells transfected with the DNAs indicated on the Figure were fractionated on a 1% formaldehyde-agarose gel blotted onto nitrocellulose and probed with ^{32}P-labelled 0.6-kb *Bgl*II-*Sst*I *tk* DNA. LAT3 is an LATK⁺ stable cell line obtained after transformation of LATK⁻ cells with pTK1. **(a)** The autoradiograph and **(b)** scanning across the 1.3-kb band.

of MoMuSV is deleted in pTK1MOE1), and with both orientations of the BPV-1 enhancer, strongly suggests that the host response does not directly involve the 'promoter' regions of the transcriptional control elements of these viruses.

Interestingly, the long term transformation assay (Figure 2) gave the same results as the short term assays for the SV40 and MoMuSV enhancers. We feel that transformation rates must reflect the initial rates of *tk* gene expression, and we

shall report on this aspect elsewhere (Lang *et al.*, in preparation). We merely wish to point out that further regulation of gene expression occurs during the establishment of long term cultures, which is reflected in gene copy number and transcriptional activity.

Although enhancer DNA sequences were initially found in viral genomes (Capecchi, 1980; Benoist and Chambon, 1981; Gruss *et al.*, 1981; Banerji *et al.*, 1981; De Villiers *et al.*, 1981), it has also been shown that similar types of sequences are present in the cellular genome (Grosschedl and Birnstiel, 1980; Conrad and Botchan, 1982). The mechanism of action of enhancers is at present unknown. However, several possibilities may be considered. Firstly, enhancers may act as chromatin organizers which may involve the generation of 'open' or 'active' chromatin structures similar to those described for viral (Saragosti *et al.*, 1980) or cellular (Stalder *et al.*, 1980) genes. Secondly, they may act by changing the superhelical density of the DNA (Smith, 1981). Thirdly, they may provide an entry site for RNA polymerase II or a polymerase-associated molecule, i.e., a sigma-like factor (Moreau *et al.*, 1981; Wasylyk *et al.*, 1983), and fourthly, they may act by binding to the nuclear matrix where viral DNA and transcriptional complexes have been found (Jackson *et al.*, 1981). These possibilities need not be independent or mutually exclusive.

The finding that viral enhancer sequences act in a host-specific manner suggests that they recognize host-cell regulatory molecules. It seems probable that the differing response of enhancers to host cell regulatory molecules may explain some of the biological effects of these viruses. In this context it will be important to determine whether diffrences in the host cell-enhancer interaction, such as have been demonstrated between species, also occur between different cell types within species. Such studies are currently underway in our laboratory.

Materials and methods

Recombinant plasmids

Recombinant vectors (Figure 1) were constructed carrying the enhancer sequences of SV40, MoMuSV and BPV-1 DNAs in pTK1, a previously described plasmid (Spandidos and Paul, 1982) containing a 3.5-kb *Bam*HI fragment of HSV-1 DNA (Wilkie *et al.*, 1979) inserted into the *Bam*HI site of pAT153 (Twigg and Sherratt, 1980). A more detailed description of these recombinants will be published elsewhere (Lang *et al.*, in preparation; Campo *et al.*, 1983).

DNA-mediated gene transfer

Transfections of mouse LATK$^-$ (Wigler *et al.*, 1979), rat 4 TK$^-$ (Topp, 1981) and human 143 TK$^-$ (Bacchetti and Graham, 1977) cells were carried out using the calcium phosphate technique (Graham and Van der Eb, 1973) with the following modifications. The DNA-calcium phosphate co-precipitate was added to the culture medium at a ratio of 1.0 ml co-precipitate/10 ml medium/1 x 10^6 exponentially growing recipient cells/25 cm^2 flask. After 20 h the medium was replaced with fresh non-selective medium (SF12-Flow Laboratories – containing 15% Hyclone Serum, Sterile Systems, Inc.) for an additional 24 h before selective medium containing HAT (Littlefield, 1964) was applied. The medium was changed every 3 days for up to 2 weeks before colonies were counted.

Assay of TK activity

The assay of TK activity in the presence of 0.2 mM TTP has been described previously (Wilkie *et al.*, 1979; Spandidos and Paul, 1982).

RNA analysis

RNA extraction from cells, RNA spot hybridization assays (Spandidos *et al.*, 1981) and Norther blot hybridization analysis (Spandidos and Paul, 1982) have been described in detail elsewhere. ^{32}P-labelled DNA probes with specific activities of 2 x 10^8 c.p.m./μg were made by nick-translation (Rigby *et al.*, 1977). The hybridization was performed in 5 x SSC, 50% formamide for 24 h at 42°C with 10 ng/ml probe as described (Wahl *et al.*, 1979) using 2x Denhardt's solution (Denhardt, 1966). The nitrocellulose sheets were extensively washed in 1 x SSC at 60°C and exposed to hypersensitized X-ray films at −70°C (Laskey and Mills, 1977).

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