Control of Expression of the Herpes Simplex Virus Thymidine Kinase Gene in Biochemically Transformed Cells

By ADELE EL KAREH, SAUL SILVERSTEIN* AND JIM SMILEY

Department of Microbiology, Columbia University, 701 West 168th Street, New York, New York 10032, U.S.A. and Department of Pathology, McMaster University, Hamilton, Ontario, Canada

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SUMMARY

A series of cell lines was constructed by transformation of murine LTK- cells with a family of deletion mutants of the herpes simplex virus (HSV) thymidine kinase (tk) gene. These mutants, differing in the extent of 5' sequence flanking the coding region for tk, varied in the frequency with which they were able to convert tk- cells to the tk+ phenotype. Converted cell lines were analysed for tk DNA sequences, tk mRNA sequences, the 5' terminus of tk-specific transcripts and for their ability to respond to a signal provided in trans by infecting tk- virus (transactivation). The results of these analyses reveal that transformation efficiency correlates inversely with the extent of 5' flanking information. Thus mutants retaining less than 109 bp of 5' sequences transform less efficiently than those that retain at least 109 bp. Cell lines established by transformation with mutants retaining the proximal 109 bp contain relatively few copies of tk DNA whereas those which arose as a result of transformation with mutant DNA containing less than 109 bp generally contained multiple copies of tk DNA. Analyses of tk-specific transcripts revealed that cell lines derived from plasmids that transformed efficiently synthesized an mRNA which was indistinguishable by its size or 5' end from infected cell mRNA. Cell lines established by plasmids that were inefficient at transformation accumulated truncated mRNAs that initiated at aberrant start sites. The presence of the 5' 109 bp block was required for transformants to increase the level of tk mRNA and enzyme when infected with a tk- deletion mutant of HSV. We also show that transactivation does not alter the initiation site of the tk mRNA synthesized by transformants.

INTRODUCTION

DNA-mediated gene transfer (transformation) has been successfully used to identify a unique BamHI restriction endonuclease fragment of herpes simplex virus type 1 (HSV-1) DNA that contains the structural and regulatory sequences which govern thymidine kinase gene (tk) expression (Wigler et al., 1977). Analysis of TK+ transformants isolated after exposure to tk-containing DNA and selection in HAT medium, demonstrated that these cells synthesized virus-specified TK enzyme and in general contained only a single copy of tk DNA (Pellicer et al., 1978). The advent of molecular cloning has permitted the elucidation of the complete nucleotide sequence of the gene and the nucleotides that flank it (McKnight, 1980; Wagner et al., 1981). Analyses of a series of ordered 5' deletion mutants, all of which terminate at a common 3' HindIII site, have revealed the presence of at least two control regions 5' to the natural tk mRNA cap site (McKnight et al., 1981). These regions quantitatively control the expression of tk DNA in a Xenopus laevis transcription system and affect the efficiency of converting LTK- cells to the TK+ phenotype following transformation with these mutant DNAs.

We have examined the capacity of these deletion mutants and a variety of naturally occurring 'mutants' that were generated by subcloning unique fragments of the intact BamHI fragment for the frequency with which they transform cells in culture. We then investigated three
biochemical features of these transformed cells: the number of insertions in stable transformants, the size of tk-specific mRNAs accumulating in them and the capacity of transformants to increase the level of TK activity in response to a signal provided in trans by an infecting TK- virus (transactivation).

Transactivation of HSV tk information in transformed cells has previously been shown to result from expression of an immediate-early (z) polypeptide (Garfinkle & McAuslan, 1974; Leiden et al., 1976; Kit et al., 1978a). The very elegant studies by Preston have demonstrated that this regulatory protein (VMW 175) is required to regulate in a positive fashion the expression of nearly all of the later classes of HSV proteins (Preston, 1979a, b). Recently, Post et al. (1981) have provided evidence that control of expression of the tk gene in transformed cells is regulated by the sequences that are 5' to the structural information of the gene. In this study we demonstrate that the amount of 5' sequences present in transforming DNA governs the frequency of conversion of TK- cells to the TK+ phenotype, affects the copy number of tk DNA in transformants and the size of tk mRNA synthesized in these cells. We also demonstrate that the increase in TK enzyme activity that occurs in response to infection results from the accumulation of tk mRNA and that at most 109 nucleotides of 5' flanking information are required for this response.

METHODS

Cells and viruses. Murine LTK- APRT- cells were used as recipients in our transformation studies and for plaque purification of TK- virus strains. They were grown and maintained as described by Wigler et al. (1977). Vero cells were used to propagate virus stocks and to titrate virus as previously described (Nishioka & Silverstein, 1977).

Two TK- virus strains were used in this study: D2, a deletion mutant constructed by reverse marker rescue and lacking 875 bp of tk coding information (Smiley, 1980), and B2006, a TK- mutant originally isolated by Dubbs & Kit (1964). D2 produces no tk mRNA or TK protein. B2006 transcribes a tk-like mRNA, but there is no detectable TK activity or protein synthesized in cells infected with this virus. We have used the abbreviations TK to refer to thymidine kinase protein, TK+ to indicate the phenotype of cell lines and tk to indicate genotype or when referring to nucleic acid (DNA or mRNA) coding for thymidine kinase.

Transformation. Cells were transformed to TK+ using either purified restriction endonuclease fragments of tk-containing DNA or fragments subcloned in pBR322. The DNA was frequently linearized by digestion with SauI which does not cleave within tk DNA and recognizes only a single site in pBR322. The deletion mutants were a gift from Steve McKnight and their properties have been previously described (McKnight & Gavis, 1980). Transformation was done as previously described using either 50 or 100 ng of tk-containing DNA and 20 µg of LTK- carrier DNA in each dish (Wigler et al., 1979a). Colony formation was scored following formaldehyde fixation and Giemsa staining after 15 to 20 days of growth in selective medium. Clones were picked for analysis using sterile cloning cylinders and then expanded by continuous growth under selective pressure.

DNA extraction and blot hybridization. All transformed cell lines were re-cloned and DNA was isolated at passage 5 as previously described (Pellicer et al., 1978). For blot hybridization 10 µg of transformed cell DNA was digested to completion with the appropriate restriction endonuclease under the conditions specified by the supplier (Bethesda Research Laboratories). The digested DNA was electrophoresed on horizontal agarose slab gels in TBE (50 mM-Tris, 50 mM- H3BO3, 1 mM-EDTA pH 8.3) at 50 to 80 V overnight. The DNA in the gels was then depurinated (Wahl et al., 1979) and transferred to nitrocellulose paper for Southern blot hybridization as previously described (Wigler et al., 1979b). 32P-labelled nick-translated probes were prepared as described by Maniatis et al. (1975). Following hybridization and washing, the filters were exposed to X-ray film at -70 °C with intensifying screens.

RNA isolation and size determination. Poly(A)-containing RNA was isolated from the cytoplasm of transformed cells at the fifth passage as follows. Cells were harvested in cold PBS (phosphate-buffered saline) and resuspended in TCM (20 mM-Tris-HCl pH 8.3, 3 mM-CaCl2, 2 mM-MgCl2) containing 0.5% Nonidet P40 for 20 min on ice. The nuclei were removed by centrifugation and the cytoplasm was brought to 0.5% SDS and extracted twice with phenol and twice with chloroform:isoamyl alcohol (24:1). NaCl was added to 0.3 M and the RNA was concentrated by addition of 2 vol. ethanol. Poly(A) RNA was selected by affinity chromatography on oligo(dT)-cellulose as previously described (Nishioka & Silverstein, 1977).

The size of tk-specific poly(A)-containing RNA was determined by electrophoresis through agarose-formaldehyde gels, transfer to nitrocellulose and hybridization with 32P-labelled DNA, as previously described (Ostrander et al., 1982).

Transactivation. TK+ transformants were seeded at 5 × 10⁵ cells/60 mm tissue culture dish in medium containing HAT (15 µg/ml hypoxanthine, 1 µg/ml aminopterin and 5 µg/ml thymidine). The next day the cells were harvested in cold PBS and RNA was isolated as described above. The RNA was size fractionated by electrophoresis through agarose-formaldehyde gels and the RNA content of each band was measured using a phosphorimager.
were infected at various multiplicities of infection for 90 min at 37 °C. The infected cells were then overlaid with medium containing 1% calf serum and harvested by scraping at intervals post-infection. The cells were centrifuged and washed in ice-cold PBS and the resulting pellets were frozen at −70 °C. The cell pellets were thawed and the amount of protein and the level of TK activity for each sample was determined in duplicate. Protein concentration was determined by the method of Bradford (1976) using commercially available reagents (Bio-Rad Laboratories). TK activity was measured as described by Lin & Munyon (1974). Re-examination of these cell lines after 6 months in culture revealed no change in the level of tk induction when compared to control lines containing only a single copy of the tk gene. For analysis of tk mRNA in transactivated cells, replica cultures of each transformant were prepared and, as the cultures were approaching confluence, the cells from a representative culture were counted and the remaining cultures were infected with 2-5 p.f.u./cell of D2. At intervals post-infection, cells were harvested and the RNA extracted and analysed for the size of tk-specific transcripts by Northern blot hybridization.

Preparation of single-strand probes. Uniformly labelled, single-strand probes for determining the polarity of transcription and the 5' end of tk transcripts were prepared after subcloning specific fragments of tk DNA into the poly linker contained in the replicative form of bacteriophage M13 mp9. Briefly, the 875 bp PstI and 825 bp BamHI–BglII fragments from pTK5 (see Fig. 1) were isolated and inserted into the unique PstI and BamHI sites. These DNAs were transformed into competent Escherichia coli JM103. The entire reaction mix was plated in the presence of X-Gal and recombinant phage were identified on the basis of their ability to form clear plaques. Representative phage were isolated and their orientation determined by hybridization of single-stranded phage DNA to end-labelled tk DNA probes. High specific activity probes were prepared by hybridizing the universal primer (P-L Biochemicals) to single-stranded phage DNA and extending the primer with the Klenow fragment of DNA polymerase I in the presence of all four 32p-labelled deoxyribonucleotide triphosphates. Primer-extended DNAs were cleaved outside the tk coding segment with HindIII and the shorter, single-stranded probe was isolated by electrophoresis through a 7 M-urea–5% acrylamide. 32p-labelled single-stranded DNA was located following a brief exposure (10 s) to X-ray film. The band was excised and the labelled DNA was eluted by incubation in a small volume of 0.4 M-NH4CH3COOH, 0.2% SDS at 65 °C for 12 to 16 h. After filtration through a cellulose acetate filter to remove acrylamide, the DNA was used as a probe to determine the polarity of transactivated tk transcripts or in S1 nuclease mapping experiments.

RESULTS

Sequences required for proficient transformation

Previous experiments in our laboratory and others (Colbere-Garapin et al., 1979; Enquist et al., 1979; Wilkie et al., 1979; Kit et al., 1980) showed that the PvuII site at −197 is the restriction endonuclease site closest to the 5' boundary of the coding sequences for the tk gene that does not destroy efficient expression. Sequence data reveal the presence of a termination codon (UAG) at nucleotide 1438 and a unique SmaI site located at nucleotide 1415 (McKnight, 1980; Wagner et al., 1981) (Fig. 1). Transfection with a fragment bounded by PvuII at the 5' end and SmaI at the 3' end results in inefficient conversion of TK− cells (Table 1; Kit et al., 1980). We tested a number of unique fragments for their ability to convert TK− cells to the TK+ phenotype (Table 1). Individual colonies were picked and analysed by blot hybridization to determine whether the integrity of the transforming fragment was maintained. Analysis of the Southern blots revealed that transformants isolated after exposure to tk DNA-containing sequences 5' to the EcoRI site, tended to have relatively few copies of integrated tk information. Those TK+ cell lines derived following transformation with DNA containing the sequences at or 3' to the EcoRI site contain multiple copies of the transforming DNA (data not shown). These experiments suggest a role for the sequences between the sites bounded by EcoRI at −78 and PvuII at −197 for proficient conversion of TK− cells. Attempts to identify restriction endonuclease sites in transformed cell DNA that were proximal to the 5' and 3' ends of the transforming fragment indicated that the naturally occurring penultimate restriction endonuclease site was invariably lost. This nibbling at the termini of linearized DNA has been observed before (Lai & Nathans, 1974; Wigler et al., 1979b). Therefore, we could not identify the ends of the integrated tk DNA when linearized DNAs were employed to convert TK− cells to the TK+ phenotype.

The direction of transcription and the boundaries of the mRNA for tk have been previously determined (McKnight, 1980; Smiley et al., 1980). These experiments suggested that the sequences controlling transcription of this gene are located 5' to the cap site of the message and 3'
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Fig. 1. Restriction endonuclease map of the 3-6 kb BamHI tk fragment from HSV-1. The solid bar above the line identifies the boundaries of the deleted PstI fragment in the D2 mutant virus (Smiley, 1980). *The KpnI site is not present in the wild-type fragment, but is present in all of the 5' deletion mutants (McKnight & Gavis, 1980). *Identifies the HindIII linker present at the 3' end of each 5' deletion mutant (McKnight & Gavis, 1980). The following abbreviations are used to identify restriction endonuclease sites: B = BamHI, Bg = BglII, E = EcoRI, Hf = HinfI, Hn = HindIII, K = KpnI, Ps = PstI, Pv = PvuII, Sc = SacI, Sm = SmaI.

The nucleotide sequence of the 5' region of the HSV tk gene as determined by McKnight (1980) is shown below the map. The 5' ends of the deletion mutants used in this study are noted above the sequence.

Sequence complexity of cells transformed with mutant DNAs

The process of transformation can involve cutting and rejoicing of, or recombination between, input DNA molecules (Perucho et al., 1980; Folger et al., 1982). A consequence of these reactions is that each transformant contains tk DNA at a unique location (Robins et al., 1980).
Table 1. Transformation efficiency of tk-containing DNAs

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Cloning vector</th>
<th>5' deletion*</th>
<th>No. of colonies/plate</th>
<th>Average no. colonies/plate/50 ng DNA</th>
<th>Colonies/μg DNA</th>
<th>T.E.†</th>
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<td>129-5</td>
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<td>pBR322</td>
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<td>-</td>
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<td></td>
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<td>80</td>
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* 5' deletion refers to the end-point of the fragment in terms of distance to the 5' end of wild-type tk mRNA in base pairs.
† T.E., the transformation efficiency is determined from the ratio of colonies per μg mutant DNA/colonies per μg pTK5 DNA.
‡ pTK5 is a recombinant containing the 3.6 kb BamHI tk fragment from HSV-1 F.
§ Unique 1-6 kb Smal fragment isolated from pTK5.
¶ pPvuII is a recombinant containing the 2.0 kb PvuII tk fragment.
¶ pMOE is a recombinant plasmid containing the 2.4 kb EcoRI fragment.
** pBglII is a recombinant plasmid containing the 2.8 kb BglII/BamHI fragment from pTK5.
††† Smal cleaves within the sequences that code for the carboxy terminus of the protein (Wagner et al., 1981).
‡‡‡ N.D., Not determined.

Therefore, we examined the complexity of tk DNA in transformants derived by transfection with DNA from the various deletion mutants. Three assays were employed to examine the number of integration events and to evaluate the integrity of the transforming DNA. First, the minimum number of integration events was determined by cleaving DNA from transformants with an enzyme that does not cut within the tk-containing fragment. Then we analysed transformants containing multiple copies of tk DNA for the presence of tandem arrays of integrated sequences by digesting the DNAs with either BamHI or HindIII and comparing their hybridization patterns. Finally, to determine the integrity of the integrated sequences, DNAs from transformants were digested with both HindIII and BamHI and the sizes of the digestion products were determined by Southern blot hybridization.

DNA from cell lines converted to the TK⁺ phenotype by exposure to mutant DNAs terminating at +1, -32, -46, -53, -56, -85, -109, -148, -180, -215 and -418 respectively was digested with BamHI and analysed by Southern blot hybridization. Analysis of the autoradiogram revealed that transformants derived following exposure to mutants deleted...
past − 109 generally contained multiple copies of tk DNA, whereas those derived from mutants retaining ≥ 109 bp 5' to the cap site tend to result from a limited number of integrations of tk DNA (Fig. 2). There are cell lines that do not respect these general principles (see Fig. 2, lanes b and i); however, they are exceptions that reflect the biological variation of the system.

DNAs from representative cell lines were analysed by blot hybridization to determine how the sequences in transformants containing multiple copies of tk DNA were arranged in the host chromosome. The experimental design that we adopted was to cleave DNA from multiple copy transformants with either BamHI or HindIII and then to compare their hybridization profiles. If the multiple copies arose as a result of tandem integration events at a single site, then the hybridization profiles should be conserved. If, however, the multiple copies arose as a result of integration at multiple sites then the two digestions should result in different patterns. Therefore, DNA from cell lines converted as a result of transfection with a mutant gene terminating at either − 56 or +1 was examined by this protocol. The autoradiogram revealed that in two of the four lines examined (Fig. 3, lanes e, f and g, h) the hybridization profiles were conserved to a large extent. This result is consistent with tandem integrations at a restricted number of sites. These arrays might be accounted for by ligation of donor termini or recombination between donor molecules prior to integration. In two other lines (Fig. 3, lanes a, b and c, d) the majority of the bands differ in mobility. We postulate that this pattern arises from interactions between donor molecules and carrier DNA resulting in multiple integration events that are most probably restricted to a single chromosomal site (Roberts & Axel, 1982; Robins et al., 1981). Clearly, not all of the copies are in tandem even in those cell lines that have a preponderance of comigrating bands.
Fig. 3. Analysis of multiple copy transformants by Southern blot hybridization. 10 µg of DNA from each of five different cell lines was digested with BamHI and HindIII and loaded in adjacent lanes for analysis by blot hybridization. Numbers above the lanes indicate the distance from the cap site to the synthetic BamHI linker located at the 5' end of the gene. The DNAs analyzed, and the enzyme they were digested with are as follows: (a) AS9-1, BamHI; (b) AS9-1, HindIII; (c) A50S2-2, BamHI; (d) A50S2-2, HindIII; (e) AS9-2, BamHI; (f) AS9-2, HindIII; (g) AS2-3, BamHI; (h) AS-2, HindIII.

Therefore, the tk copy number in transformants correlated inversely with the transformation efficiency of donor DNA. DNAs that were highly proficient resulted in transformants with low copy number whereas those DNAs which inefficiently donated tk tended to convert TK− cells only as a result of numerous integration events; these results corroborate the studies of Zipser et al. (1981).

Each mutant template has a conserved 3′ HindIII site and varying amounts of 5′ flanking sequence marked by a BamHI site; therefore, it was possible to cleave DNA isolated from individual transformed cell lines with BamHI and HindIII and to examine the integrity of the donor DNA. If the hybridizing band was identical in size to the tk fragment liberated from the cloned deletion mutant, we considered the termini to have been conserved in the transformed cell. The results of this experiment revealed that transformants derived from mutant DNAs retaining sequences at or 5′ to −109 contained a limited number of tk-specific sequences which were identical in size to the tk information in the transforming DNA. A similar analysis of DNA from transformants deleted past −109 demonstrated that they contained numerous copies of tk DNA of various sizes (Fig. 2 and 4). The predominant hybridizing species comigrated with a BamHI/HindIII double-digested marker DNA that was used to convert the cell. Thus, we concluded that the sequences between the natural 5′ end of the tk mRNA and −109 represented the maximal amount of regulatory information necessary to ensure proficient conversion of TK− cells to the TK+ phenotype. Transformants that arose as a result of exposure to DNA
Fig. 4. Mapping the 5' and 3' boundaries of transforming DNA. 15 μg of DNA from each transformant was digested with BamHI and HindIII, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose and probed with 32P-labelled wild-type BamHI fragment. The numbers above the lanes refer to the distance from the mRNA cap site to the synthetic BamHI site present at the 5' end of each deletion mutant. The numbers on the side refer to the sizes of the BamHI/HindIII digestion products of deletion mutant DNAs electrophoresed along with the digested DNA from transformants as size markers. The markers were visualized, after staining with ethidium bromide, by u.v. irradiation. DNA was analysed from the following cell lines: (a) AS2-3, (b) AS9-2, (c) AM10-4, (d) AM12-1, (e) AM14-1, (f) A100M11-3, (g) A100M6-4.

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retaining sequences at or 5' to -109 generally contained a few copies of tk DNA and conserved the 5' and 3' boundaries of the transforming DNA, whereas transformants derived from mutant DNAs deleted past -109 contained multiple integrated copies of tk DNA, some of which retained the boundaries of the transforming species although the majority lost one or another of the marker sites.

Size analysis of tk-specific RNA in transformants

Previous studies demonstrated that single copy transformants and HSV-infected cells accumulate tk-specific transcripts that are identical in size and maintain a common 5' cap site (McKnight & Gavis, 1980; Ostrander et al., 1982). In addition to this 1.4 kb tk-specific transcript, transformants accumulate two shorter transcripts of 1.1 and 0.9 kb that are not detected in virus-infected cells (Ostrander et al., 1982). Cytoplasmic poly(A)-containing RNA from cells transformed with mutant DNAs was analysed by northern blot hybridization to determine whether deleting 5' sequences altered the size of tk-specific transcripts. Transformants containing the sequences at or 5' to -109 accumulate a 1.4 kb tk-specific RNA which is indistinguishable in size from that synthesized in infected cells (Fig. 5 and Fig. 7a). Analysis of
HSV TK gene control

RNA isolated from transformants containing templates deleted beyond −109 revealed the presence of two predominant species of truncated mRNA which were either 1.1 or 0.9 kb in size (Fig. 5 and Fig. 7a and c). Some of the transformants that contained multiple copies of tk DNA accumulated additional species of tk RNA which differed in size from the characteristic species of RNA (Fig. 5b, Fig. 7b, lane 5 and c, lane 1). The majority of these aberrant transcripts were larger and less abundant than the truncated 1.1 and 0.9 kb tk RNAs which accumulated in transformants containing many potential templates. These transcripts may have arisen as a result of initiation from promoter sequences acquired either from the carrier DNA or from fortuitous integration adjacent to endogenous cellular promoters. The specific activity of TK enzyme in transformants rarely varied more than threefold. Thus, the basal levels of tk mRNA and enzyme in transformants correlate closely with one another. For example, transformants LHB2b (−775), AS9-2 (−56) and AM10-1 (−109) appear to contain similar levels of tk-specific mRNA on the basis of band intensity (Fig. 5, lanes a,b,c). The specific activity of TK in cytoplasmic extracts prepared from log phase cultures of these cell lines is 75, 55 and 50 ct/min/µg protein respectively. By comparison, transformants AM10-5 (−109) and AS12-5 (−46) appear to contain less tk-specific mRNA and have correspondingly lower TK activity in their cytosols (20 and 30 ct/min/µg protein respectively). Finally, these results suggested that the block of sequences between the cap site and −109 are involved in qualitatively regulating the initiation site of tk-specific mRNA in transformed cells.

Transactivation of endogenous templates

Cells converted to the TK+ phenotype as a result of infection with u.v.-irradiated virus or transformation with tk-containing DNA are subject to transactivation of the endogenous tk
Fig. 6. Kinetics of transactivation. Transformants were infected with 10 p.f.u./cell of B2006. At the
times indicated post-infection, cells were harvested and the specific activity of TK determined. The
points represent the average of three samples. (a) □, LTK-APRT-; ●, AM14-4; △, AM14-1; △,
AM14-3; ○, AM14-2. (b) □, AM12-5; ●, AM12-4; △, AM12-1; ○, AM12-3. (c) □, AM10-4; ●,
AM10-2, △, AM10-3; ○, AM10-1. (d) □, AS2-3; ●, AS9-2; △, AS9-3.

The gene as a consequence of infection by TK- virus (Garfinkle & McAuslan, 1974; Kit et al.,
1978a; Leiden et al., 1976; Lin & Munyon, 1974; Post et al., 1981; Wilkie et al., 1979; Zipser et
al., 1981). Transactivation requires expression of an immediate-early virus gene product,
VMW175 (ICP4), whose coding sequence maps partially within the repetitive sequences that
flank the short unique region of the virus genome (Knipe et al., 1979; Watson et al., 1979).
Wilkie et al. (1979) demonstrated that cells transformed by the 2.0 kb cloned PvulI fragment that
spans the tk gene can be transactivated. These transformants responded to infection with TK-
virus by increasing the specific activity of TK by 1-5- to 10-fold. The specificity of this response
was later demonstrated to reside within the adjacent 5' DNA sequence (Post et al., 1981). To
identify the sequences which lie 5' to the mRNA cap site that are required for transactivation,
transformants derived by transfection with defined DNA sequences were infected with a TK-
mutant of HSV-1 (B2006) that fails to synthesize any detectable TK (Kit et al., 1978b). At
intervals post-infection, the specific activity of TK was measured by preparing cytosol extracts
from duplicate cultures and assaying each sample in duplicate. The results of a typical kinetic
analysis are shown in Fig. 6 and are summarized for a number of transformants in Table 2. The
vast majority of transformants derived by transformation with DNA that retained the sequences
at or 5' to -109 could be transactivated. They showed levels of TK enzyme that were 4- to 10-
fold above their uninfected counterparts. When a transformant was unable to be transactivated,
we were usually able to correlate this lack of response to an alteration to the 5' sequences as
**HSV TK gene control**

Table 2. Transactivation of TK⁺ transformants

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Nucleotides from the 5' end of tk mRNA</th>
<th>Transactivation</th>
<th>Transformant</th>
<th>Nucleotides from the 5' end of tk mRNA</th>
<th>Transactivation*</th>
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<td></td>
<td>ASma-1,2,3,4(6)†</td>
<td>-460</td>
<td>NC</td>
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</tbody>
</table>

* Transactivation = sp. act. of TK at 12 h post-infection/sp. act. of TK in mock-infected cells.
† NC, No change in TK activity in response to infection; transactivation = 1.0.
‡ (1) Derived as described in Pellicer et al. (1978), (2) derived by transformation with the 2.0 kb PvuII fragment from pTK-5, (3) transformed with the HindII/BamHI 2.8 kb fragment from pTK-5, (4) transformed with the BglII/BamHI 2.8 kb fragment, (5) transformed with a cloned BglII/BamHI 2.8 kb fragment, (6) transformed with the PvuII/SmaI fragment from pTK5, (7) transformed with the SmaI 1.6 kb fragment from pTK5.

**Table 2. Transactivation of TK⁺ transformants**

Determined by loss of the BamHI site marking the 5' boundary (data not shown). Cells transformed with mutant DNAs deleted 3' to -74 failed to be transactivated as judged by this assay (Fig. 6 and Table 2). Transformants derived following exposure to mutant DNAs terminating between -74 and -95 frequently showed a low level (1-5- to 2-fold) of stimulation in this transactivation assay.

To determine whether transactivation resulted from increased utilization of tk mRNA or an increase in the amount of tk mRNA, the steady state level of mRNA in a single copy transformant (Pellicer et al., 1978) was analysed by northern blot hybridization at 6 and 12 h post-infection with D₂, a tk⁻ deletion mutant of HSV-1 (Smiley et al., 1980). A cloned 875 bp PstI fragment was used as a hybridization probe in these experiments because these sequences are absent from the genome of the infecting virus. Identical amounts of poly(A)-containing cytoplasmic RNA from mock-infected and infected cells were analysed. The hybridization signal increased in intensity as a function of time post-infection (Fig. 7a). Thus, the increased amounts of TK reflect an accumulation of tk mRNA sequences. Mock-infected cells show no increase in the amount of tk-specific mRNA during the same time period.

We next examined the levels of tk-specific mRNA that accumulated after infection of transformants constructed by transfection with the various deletion mutant DNAs. Previous studies demonstrated that when transformants containing mutant templates terminating at +1,
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Fig. 7. Blot hybridization analysis of RNAs from transactivated cells. Poly(A)-containing RNA was isolated from transformants infected with 2.5 p.f.u./cell of D$_2$. Identical amounts of poly(A)-containing RNA were analysed from each transformant as described in the legend to Fig. 6. (a) 2 μg of poly(A) RNA from (1) 6 h infected, (2) 12 h infected or (3) mock-infected LHB2b cells. (b) 2 μg of poly(A) RNA from transformant AS2-3, (1) mock-infected or (2) 8 h post-infection. 4 μg of poly(A) RNA from A100M20-3, (3) mock or (4) 8 h post-infection; AS9-2, (5) mock, (6) 12 h post-infection, (7) a shorter exposure of (6). (c) 4 μg of poly(A)-containing RNA from A3-1, (1) mock, (2) 8 h post-infection; AM10-5, (3) mock, (4) 8 h post-infection. In (a) the 667 bp BamHI–SacI fragment was used as probe; in (b) and (c) the 875 bp PstI fragment was used.

Transformants derived following exposure to mutant DNAs whose 5' boundary is between –74 and –95 respond in an intermediate way when compared to wild-type genes in the transactivation assay. Therefore, tk-specific RNA from a transformant constructed by transfection with a mutant terminating at –80 was analysed post-infection with D$_2$ and compared with mock-infected RNA. This analysis revealed that numerous previously undetected RNA species appear in response to infection (Fig. 7c).

When RNA from a transformant containing a mutant template retaining >109 bp 5' to the mRNA cap site was analysed after infection with D$_2$, it showed elevated levels of the 1.4 kb tk mRNA (Fig. 7c, lanes 3 and 4).

Origin of transactivated tk-like mRNAs

The previous experiments revealed the presence of increased amounts of new species of RNA that annealed to tk DNA (Fig. 7b; c, lanes 1 and 2). Many of these cell lines contain multiple copies of tk DNA and it is likely that more than one of these templates is being transcribed. Because the basal level of TK in cells is not higher than in single copy transformants, and does not increase in response to transactivation, it is unlikely that these additional transcripts code for functional TK. The derivation of these tk-like RNAs is uncertain. To determine whether these aberrant RNAs, which appear in response to infection, arise from transcription of the coding or the non-coding strand of tk DNA, single-stranded probes specific for coding and non-coding strands were hybridized to Northern blots of RNAs from mock-infected and D$_2$-infected transformants containing the wild-type and the –56 template (Fig. 8a, b and c). Single-stranded
Fig. 8. Polarity of aberrant transactivated RNAs. Total, cellular poly(A)-containing, RNA was isolated from mock-infected and D2-infected transformants and analysed by Northern blot hybridization. (a) The 875 bp, double-stranded PstI fragment was nick-translated to high specific activity and used as probe: (1) LHB2b mock, (2) LHB2b-infected, (3) AS9-7 mock, (4) AS9-7-infected. (b) 32P-labelled DNA complementary to the non-coding strand of the 875 bp PstI fragment was prepared as described in Methods and used to probe blots containing RNA from (1) HSV-1 F-infected cells, (2) LHB2b mock, (3) LHB2b-infected, (4) AS9-7 mock, (5) AS9-7-infected. (c) 32P-labelled DNA complementary to the tk mRNA was prepared and used to probe a blot identical to that shown in (b). RNA was from (1) HSV-1 F-infected cells, (2) LHB2b mock, (3) LHB2b-infected, (4) AS9-7 mock, (5) AS9-7-infected.

DNA with tk mRNA polarity did not hybridize to poly(A)-containing RNA from mock-infected, wild-type or mutant template (-56) transformants, D2-infected transformants or to wild-type HSV-1-infected cells (Fig. 8b). Single-stranded probe complementary to tk mRNA hybridized to RNA from all of these sources (Fig. 8c). Therefore the aberrant transcripts that accumulate in response to transactivation with D2 are synthesized from the tk coding strand.

Where do these aberrant transcripts initiate? The appearance of transcripts that are homologous to and longer than authentic tk mRNA suggests that some of these may have arisen as a result of initiation at a site far upstream from the body of the tk mRNA. To explore this possibility, we attempted to map the 5' ends of tk-like RNAs from uninfected and infected transformants. The 5' BamHI–BglII fragment from the BamHI-digested tk wild-type DNA was cloned in bacteriophage M13 mp9 at the unique BamHI site in the replicative form. A virus containing the non-coding strand was identified and used as a template to synthesize uniformly labelled DNA that was complementary to tk mRNA. To determine whether the 5' end of the tk RNAs synthesized after infection extended past the normal initiation site (Fig. 1), this uniformly labelled DNA was used as a hybridization probe in S1 nuclease protection experiments. We reasoned that if RNA from a transformant protected a larger portion of the probe than authentic tk mRNA did, then this would suggest that these transcripts arose as a result of utilization of 'acquired' initiation sites. Accordingly, RNA from cells infected with HSV-1 [F] or HSV-1 [D2] was isolated and hybridized to the 32P-labelled single-stranded probe and the size of fragment protected from digestion by S1 nuclease determined by electrophoresis through a 7 M-urea–10% acrylamide gel. The results of this experiment show that wild-type
Fig. 9. S1 nuclease mapping of tk mRNAs. Total cellular RNA was extracted from cells, infected with HSV-1 D₂ or mock-infected, and hybridized to uniformly ³²P-labelled single-stranded DNA including the 5' end of the tk gene from the BglII site at +56 to the upstream BamHI site at -775. Unhybridized probe was removed by digestion with S1 nuclease and the protected fragment was analysed by electrophoresis through a 10% acrylamide, 8 M-urea gel at 1500 V until the bromophenol blue dye reached the bottom of the gel. The gel was exposed to X-ray film at -80 °C with an intensifying screen. The lanes contained RNA from (a) HSV-1 D₂-infected cells, (b) HSV-1 F-infected cells, (c) AS12-5, mock-infected, (d) AS12-5 infected, (e) AS9-4, mock-infected, (f) AS9-4, infected, (g) AS9-7, mock-infected, (h) AS9-7, infected, (i) LHB2b mock-infected, (j) LHB2b, infected, (k) HSV-1 F-infected cells. The numbers at the side of the figure represent the distances that end-labelled, HpaII-digested, pBR322 marker fragments migrated.

tk mRNA protects a 56-base fragment, while RNA from cells infected with D₂ fails to protect the probe (Fig. 9a, b). Poly(A)-containing RNA isolated from mock-infected or D₂-infected LHB2b cells, a single copy transformant (Pellicer et al., 1978), protected the same 56-base fragment (Fig. 9i, j). Thus, transactivated tk RNAs initiate at the same site as transformed cell mRNA and this transcript is indistinguishable from bona fide HSV-1 tk mRNA. RNA isolated from transformants AS12-5 (−46) and AS9-4 and AS9-7 (−56) protected fragments larger than 56 bases (Fig. 9c, e, g). After infection AS9-4 and AS9-7 accumulate increased levels of
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aberrantly sized tk-like RNAs some of which are longer than authentic tk (Fig. 7 and 8). RNAs from these cells protect fragments of single-stranded probe that are 56 bases or longer (Fig. 9f, h). Because of the nature of the probe, this can only occur if some of these transcripts initiate upstream from the normal tk start site. RNA from AS12-5 infected with D2 protects a 102-base fragment (Fig. 9d). The presence of a fragment of this size suggests that some of the RNAs homologous to the tk probe were initiated from a point 5' to the 46 bp non-coding region of this mutant template.

DISCUSSION

We have constructed TK + cell lines using a series of defined deletion mutants of the herpes simplex virus thymidine kinase gene (McKnight & Gavis, 1980) to study what effect deleting specific sequences flanking the 5' end of this gene would have on its expression in the transformed cell. In this study we examined the role of these sequences by measuring the transformation efficiency of these mutant templates and by examining the ability with which cell lines transformed with these defined templates could be transactivated in response to infection with tk - virus. The transactivation studies included measurements of enzyme levels and analyses of RNAs with homology to tk. Because establishment of transformed cell lines is a complex process which can involve ligation of donor DNA to carrier DNA (Perucho et al., 1980), recombination between donor molecules which can result in mutation and activation or inactivation of genes (Folger et al., 1982) and insertion at different sites in each transformed cell line (Robins et al., 1981), it was necessary to survey several independent transformants derived from the same DNA source.

Measurements of the efficiency with which defined mutants converted TK - cells to the TK + phenotype confirmed the results of McKnight et al. (1981). Specifically, we demonstrated that a block of 109 bp, located immediately adjacent to the 5' end of the sequences that code for tk mRNA, was required for proficient conversion. When mutants retaining these sequences were employed as donors, the transformation efficiency was similar (≥ 50%) to that found with wild-type DNA. This same block of 109 bp was shown to contain all of the information necessary for proper transcription of the transformed gene and to permit virus-specified transactivating signals to alter the expression of this locus. Southern blot hybridization analysis of the tk gene in TK + transformants demonstrated that cell lines derived by exposure to mutant DNAs that retained the 109 bp block generally resulted from the integration of one or only a few copies of tk DNA. In contrast, deletion mutant DNAs that terminated 3' to position -109 were inefficient transformers and cell lines established from these DNAs contained multiple copies of these mutant genes. These results support those of Zipser et al. (1981) who, in surveying a limited number of cell lines constructed by transformation with linker insertion mutants, concluded that the copy number of tk genes correlated inversely with the transformation efficiency of the mutant DNA. Parenthetically, we noted that all transformants, regardless of the number of integrated templates and whether templates were wild-type or mutant, contained very similar levels of TK activity. It appears that transformants selected in HAT require a minimal basal level of enzyme activity for survival.

Analysis of transcription products from both deletion and 'linker scanning' mutants in a Xenopus oocyte system has demonstrated the complex nature of the 5' regulatory region of the HSV tk gene (McKnight et al., 1981; McKnight & Kingsbury, 1982; McKnight, 1982). These studies have revealed that constitutive transcription in this system requires the presence of three domains that map to non-overlapping sequences at -12 to -29, -46 to -59 and -80 to -105 in the 5' regulatory region of this gene. Alterations to, or deletion of, these sequences result in changes in the initiation site for tk mRNA (-12 to -29), or the quantity of tk mRNA synthesized (-46 to -59 and -80 to -105). Our studies on the tk mRNAs that are constitutively expressed in transformants that have revealed that deletion of the sequences 3' to -109 alters the size of the predominant transcript that accumulates in these cells. Transformants that contain wild-type templates, i.e. 5' terminus ≥ position -109, predominantly synthesize a transcript that is indistinguishable in size and initiation size from authentic tk mRNA which accumulates in virus-infected cells. Two other transcripts, 1-1 and
0.9 kb, whose initiation site(s) has not been mapped, are occasionally seen. These truncated transcripts are the same size as those detected in cells converted by DNAs retaining <109 bp of 5' sequence. Thus these studies, using transformed cells, demonstrate that the 109 bp block contains all of the elements required for constitutive expression in murine cells. Deletion from this block alters the size of the tk transcript synthesized in transformed cells. A similar conclusion was reached by Minson et al. (1982) when they examined cell lines derived by transformation with sheared HSV-2 DNA for the presence of virus-specific sequences on the upstream side of the tk gene. In these studies only transactivation of the tk enzyme was measured; no attempt to examine the transactivated mRNAs was made.

The different responses of transformants, containing altered amounts of 5' sequence, to transactivation suggests that the region from 0 to -109 is composed of multiple regulatory domains. The studies of McKnight (1982), using linker scanning mutants, have demonstrated the presence of three, physically separated, domains that are required for quantitative transcription of the tk gene in Xenopus laevis oocytes. Zipser et al. (1981) have demonstrated that constitutive transcription of the tk gene can be separated from the transactivation response. These latter studies demonstrated that insertion of a XhoI linker at -9 in the tk gene decreased the frequency with which this DNA would transform tk- cells without affecting the capacity of cells transformed with this DNA to be transactivated. Insertion at -50 had the opposite effect. In this study we have demonstrated that transactivation of the tk gene in transformed cells can be differentiated into three responses. Thus, transformants retaining the sequences from 0 to -109 synthesize a wild-type tk mRNA and increase enzyme and tk mRNA levels in response to infection. The transactivated, transformed cell and virus tk mRNA have identical 5' ends. Deletion of the sequence between -80 and -109 alters the response of transformed cell lines, containing these sequences, to transactivation. These lines only marginally increase TK over the basal level found in the uninfected cell and fail to overproduce tk mRNA despite the fact that at least some of these transformants appear to synthesize wild-type tk mRNA (data not shown). Transformants constructed from mutants deleted 3' to -74 exhibit a different response after infection. These cells do not increase TK activity although they frequently exhibit increased levels of aberrantly sized transcripts that are homologous to a tk probe. S1 nuclease protection experiments demonstrate that these transcripts do not initiate at the site used by wild-type tk mRNA. In one instance, the 5' end of the transactivated transcript protected a fragment that extended to the end of the tk sequences used to convert this cell. Thus RNA from AS12-5 (-46) protected about 102 bases of probe DNA. A transcript of this size has most likely arisen as a result of initiation at a site outside the tk sequences.

The frequent appearance of numerous aberrant transcripts after virus infection suggests that some of these may have arisen as a result of initiation in adjacent cell or carrier DNA sequences. Fortuitous integration into certain sequences might result in the construction of a virus-like promoter that is activated after infection. Some of the short transcripts might have originated from other virus promoters located within the coding region of the tk gene. Previous studies from this laboratory (Ostrander et al., 1982) have revealed the presence of truncated transcripts of 900 bases that map entirely within the tk coding sequences of the gene. In addition, studies by Roberts & Axel (1982) have demonstrated that the 2.8 kbp BglII-BamHI fragment of the tk gene can encode a truncated tk RNA that will effectively convert tk- cells to the tk+ phenotype. Preston & McGeogh (1981) have shown that TK polypeptides of different lengths can be identified in infected cells. Some of these polypeptides may have resulted from secondary initiation of translation, as they have suggested, while others may result from translation of truncated species of tk mRNA that have initiated 3' to the BgII site within the gene.

The different responses that we have observed support the suggestion that the regulatory region of the tk gene is compound and physically split, i.e. physically distinct subsets of nucleotides may serve different functional roles in transcription. In support of this, one of us (J. R. Smiley) has observed that deletion of 9 bp around the EcoRI site at -79 fails to alter either transformation efficiency or the transactivation response of transformants. The complete description of the number and the composition of the domains required for the transactivation response remains to be elucidated.
We gratefully acknowledge Dr Steve McKnight of The Fred Hutchinson Cancer Center for the generous gift of the deletion mutants. These studies were supported by grants from the N.I.H., CA17477 and CA23767, to S. S. and from the MRC of Canada to J. S. S. S. is the recipient of a Research Career Development from the N.I.H., CA00491. Finally, we thank Nadirah Risbrook for typing and retyping and retyping.

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