Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6276–6280, October 1981
Cell Biology

Mapping functional domains in the promoter region of the herpes thymidine kinase gene

(herpes simplex virus type 1/*in vitro* mutagenesis/DNA-mediated transformation/gene expression)

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Communicated by James D. Watson, June 22, 1981

ABSTRACT **The cloned herpes simplex virus type 1 (HSV-1) thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) gene can be used to transform TK^- cells to a TK^+ phenotype. Transformants generated in this way express TK at a basal constitutive level that is inducible to a higher level by infection with TK^- herpes virus. We have studied the effect of mutations generated *in vitro* on both the constitutive and virus-induced expression of TK in transformants. Four *Xho* I linker insertions and two deletions in the 5′ untranscribed region of the cloned HSV-1 TK gene were generated *in vitro*. A deletion that removed all but nine base pairs of the 5′ untranscribed region virtually eliminated constitutive expression and completely prevented induction by herpes virus infection. Two of the insertions have particularly interesting properties. One, nine base pairs upstream from the cap site, inactivates constitutive expression without stopping induction. The other, 50 base pairs upstream from the cap site has the opposite effect (i.e., normal constitutive expression but no induction). Analysis of these results leads us to propose that the 5′ untranscribed region of the HSV-1 TK gene is quite complex with several functional domains having differential roles in the constitutive and herpes-induced expression of the TK gene.**

Studies of gene expression have been greatly facilitated by the use of cloned genes, which can be mutated *in vitro* and reintroduced into cells by transformation. We have used this paradigm to analyze the function of the 5′ untranscribed, flanking sequences of the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) gene. This gene is particularly amenable to such studies, and a large amount of detailed information about it has emerged from the work of many laboratories. The HSV-1 TK gene is present on a 3500-base-pair *Bam*HI fragment which has been cloned in pBR322 (1–5). The TK gene itself is located in a segment about 1400 nucleotides long which has been sequenced (3–8). The direction and initiation site of mRNA synthesis have been determined (7, 8). Sequences similar to those associated with promoters in various eukaryotic systems are present in the 5′ untranscribed region of the herpes TK gene (7, 8). The presence of a functional promoter in this region has been confirmed by McKnight (9, 10), who constructed a set of deletion mutants of the TK gene in which varying amounts of the 5′ flanking sequences were removed. The effect of these deletion mutations on TK gene expression was assayed by injecting plasmid DNA carrying them into *Xenopus* oocyte nuclei and assaying for both TK enzyme formation and mRNA synthesis. This study showed that a segment of the 5′ flanking sequence ending about 100 base pairs upstream from the cap site is sufficient for the full expression of the TK gene. Deletion mutations of the gene 95 or less base pairs upstream from the cap site inactivate the promoter and decrease TK expression by a factor of 10 or more.

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Many features of herpes TK gene expression can be studied by introducing the gene into animal cells with DNA-mediated transformation (1, 2, 11–16). This is generally done under selective conditions where only cells that have incorporated an active copy of the gene can survive. The herpes TK gene can be introduced nonselectively, however, and studies of this type have shown that any cell that incorporates a complete copy of the gene expresses it (17). This result indicates that no special features have to be selected in order for the herpes TK gene to express constitutively in animal cells. When the same gene is introduced into cells during productive herpes virus infection, its expression is not constitutive but depends on the presence of other herpes proteins. The herpes TK gene, which is an early or β gene, makes mRNA only when the products of the immediate early or α genes are present (18, 19). One α gene product in particular (a 175,000-dalton protein called VP175) is required for TK mRNA synthesis (20–23). Herpes TK genes that have been introduced into cells by transformation retain their ability to respond specifically to this herpes virus control mechanism. This is seen when transformants carrying the herpes TK gene are infected with TK^- herpes virus. After infection there is an induction of TK activity above the basal constitutive level found in uninfected transformants (3, 24). This induction is a response of the cell copy of the herpes TK gene to specific herpes signals because TK^- HSV-1 with a temperature-sensitive mutation in VP175 does not induce at the nonpermissive temperature (25).

To get more insight into the mechanism of expression of herpes TK gene, we constructed mutations in the 5′ untranscribed region of the cloned herpes simplex virus type 1 (HSV-1) TK gene. Plasmids carrying these altered genes were used to transform TK^- mouse L cells to a TK^+ phenotype. Cell lines derived from these transformations have been analyzed for TK gene copy number, basal constitutive level of TK activity, and the induction of TK activity after infection with TK^- HSV-1. Two deletion mutant plasmids and four *Xho* I linker insertion mutant plasmids have been analyzed in this fashion. The results obtained with these mutants have shown that alterations of the 5′ untranscribed region can block either constitutive or induced expression of the HSV-1 TK gene present in transformants.

MATERIALS AND METHODS

Isolation of Deletions. Circular pTK2.0 was partially cleaved with *Hae* III, and *Hin*dIII linkers were added to purified linear monomers (26). The preparation was then treated with *Hin*dIII and recyclized at 0°C with T4 DNA ligase. The plasmids with 5′ deletions called "pTK2.1" and "pTK2.4" were isolated from

Abbreviations: HSV-1, herpes simplex virus type 1; TK, thymidine kinase.

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the large set of deletion plasmids generated. The orientation of the herpes TK *Bam*HI fragment was inverted by cleavage with *Bam*HI followed by ligation. This plasmid, called "TK1.0," was used to generate p1.33.

Isolation of Insertions. Monomeric p1.33 DNA was partially cleaved with either *Hae*III or *Tha* I. Linear molecules were isolated and eight-base-pair *Xho* I linkers (Collaborative Research, Waltham, MA) were added; after cleavage with *Xho* I, the plasmids were cyclized. The approximate position of *Xho* I linker insertion was determined by restriction enzyme analysis, and the exact position was determined by DNA sequence determination.

DNA Sequence Determination. pTK2.1 and pTK2.4 were opened with *Cla* I, labeled with [α-^{32}P]dTTP by using the large fragment of DNA polymerase I, and cut with *Bam*HI. The TK fragment was purified on an agarose gel, and the sequence was determined by the method of Maxam and Gilbert (27).

Xho I linker position was determined by cutting with *Xho* I, end-labeling with [α-^{32}P]dTTP, and cutting with *Hin*dIII to produce two labeled fragments of unequal size, whose sequence was determined by the Maxam and Gilbert technique.

Transformation. DNA-mediated transformation of LTK$^-$ cells to LTK$^+$ was carried out by the method of Wigler *et al.* (1). TK$^-$ human DNA prepared from an osteosarcoma cell line (14) was used as carrier.

Thymidine Kinase Assays. Thymidine kinase was assayed as described by Wilkie *et al.* (3). Protein in the extracts is determined by the Bio-Rad protein assay dye reagent, and specific activity is defined as 0.01 pmol of thymidine phosphorylated per μg of protein per 15 min.

RESULTS

Construction of Deletion and Insertion Mutants *in Vitro*. The starting material for the construction of deletions was a hybrid plasmid containing the 3500-base-pair *Bam*HI DNA fragment of HSV-1 that codes for TK (Fig. 1, pTK2.0). This plasmid has the TK promoter adjacent to the unique *Hin*dIII site of pBR322. Monomers of pTK2.0 were linearized by partial cutting with *Hae* III, which cuts frequently and leaves a flush end. These linears were purified on agarose gels and 10-base-pair *Hin*dIII linkers were ligated to their ends. This was followed by cutting with *Hin*dIII, which cut at the unique site in pBR322 and also removed excess linker, leaving sticky ends at the site of the linearizing *Hae* III cut. When this material was religated at low DNA concentration, circular plasmids were formed that had deletions from the site of *Hae* III cutting to the *Hin*dIII site in pBR322. Restriction enzyme screening of these plasmids followed by DNA sequence determination of the region around the *Hin*dIII site led to the isolation of two plasmids with deletions in the 5′ untranscribed region of the cloned HSV-1 TK gene. These, called pTK2.1 and pTK2.4 (Figs. 1 and 2), remove all of the 5′ sequence up to 183 and 9 base pairs, respectively, before the cap site.

Deletions of the 3′ region of the TK gene were obtained in the same manner except that the starting plasmid (Fig. 1, pTK1.0) had its TK insert in the opposite orientation. One of these mutant plasmids with 3′ deletion (Fig. 1, p1.33), which removes the 3′ region of the *Bam*HI insert up to about 300 nucleotides downstream of the poly(A) addition site, was used for the generation of the insertion mutants. The deletion in p1.33 had no detectable effect either on transformation efficiency or TK activity. Insertion mutant plasmids were made in a fashion analogous to that used to generate deletion mutant plasmids except that *Tha* I was used in addition to *Hae* III to linearize p1.33, and 8-base-pair *Xho* I linkers were added rather than 10-base-pair *Hin*dIII linkers. Because p1.33 has no *Xho* I sites, cleavage with *Xho* I after linker addition led to material which, when cyclized at low DNA concentration, had *Xho* I linker insertions at the site of the *Hae* III or *Tha* I cuts (Fig. 1). From a large number of insertion mutant plasmids made in this way, four were mapped to the 5′ untranscribed region of the TK gene (Fig. 2). DNA sequence determination identified the four sites at 183, 50, 16, and 9 base pairs upstream from the cap site. Plasmids with *Xho* I linker insertion mutations at these points are called p1.33(-183), p1.33(-50), p1.33(-16), and p1.33(-9), respectively.

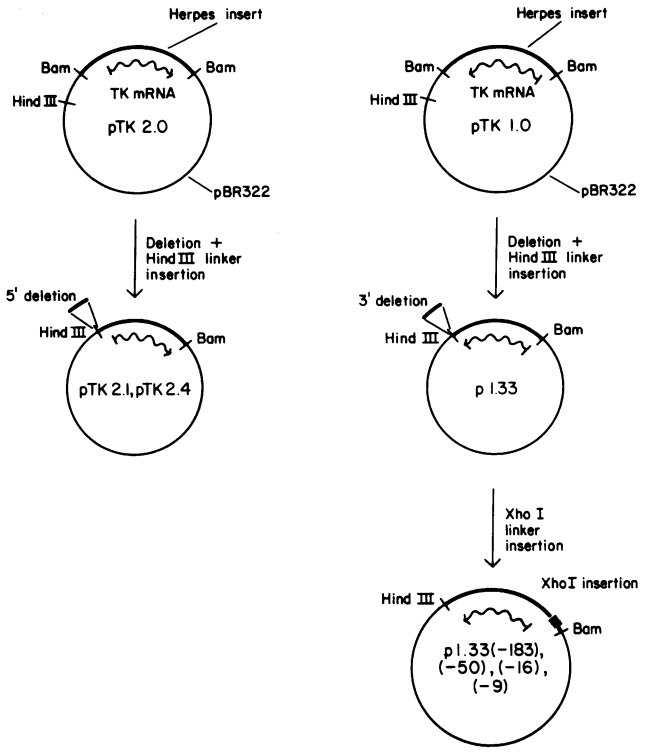

FIG. 1. Deletion and insertion construction. This figure outlines the general scheme used to generate the deletions and insertions in the 5′ untranscribed region. Only typical insertions and a deletion in the area of interest are shown but, of course, many all over the plasmid were obtained. Those of interest were found by extensive screening. The names of the plasmids of each type referred to in the text are given next to the appropriate plasmid sketch in the figure. The arrow on the line representing mRNA synthesis points in the direction of chain elongation.

Isolation of Cell Lines Transformed with Mutant TK Genes. DNA from each of the mutant plasmids was used to transform mouse LTK$^-$ cells to a TK$^+$ phenotype. Table 1 shows the transformation efficiencies observed in the experiments from which the transformed lines used in this work were obtained. The significant point made by this data is that the transformation efficiencies of the mutant plasmid DNA fall into two classes. One with a transformation efficiency approximately equal to that found in the parental plasmids, and the other with a much lower efficiency. The plasmid containing a deletion of all but nine base pairs of the 5′ untranscribed, flanking sequence (pTK2.4) is among those in the class with the low transformation efficiency, as are two of the insertion mutant-carrying plasmids [p1.33(-16), p1.33(-9).] The deletion mutation in pTK2.4 has removed the normal TK promoter. Transformation with this plasmid could only be carried out at high DNA concentrations. The transformants derived from transformation with pTK2.4 all had multiple copies of the TK gene (Fig. 3). The level of TK gene expression in transformants derived from pTK2.4 is low and about the

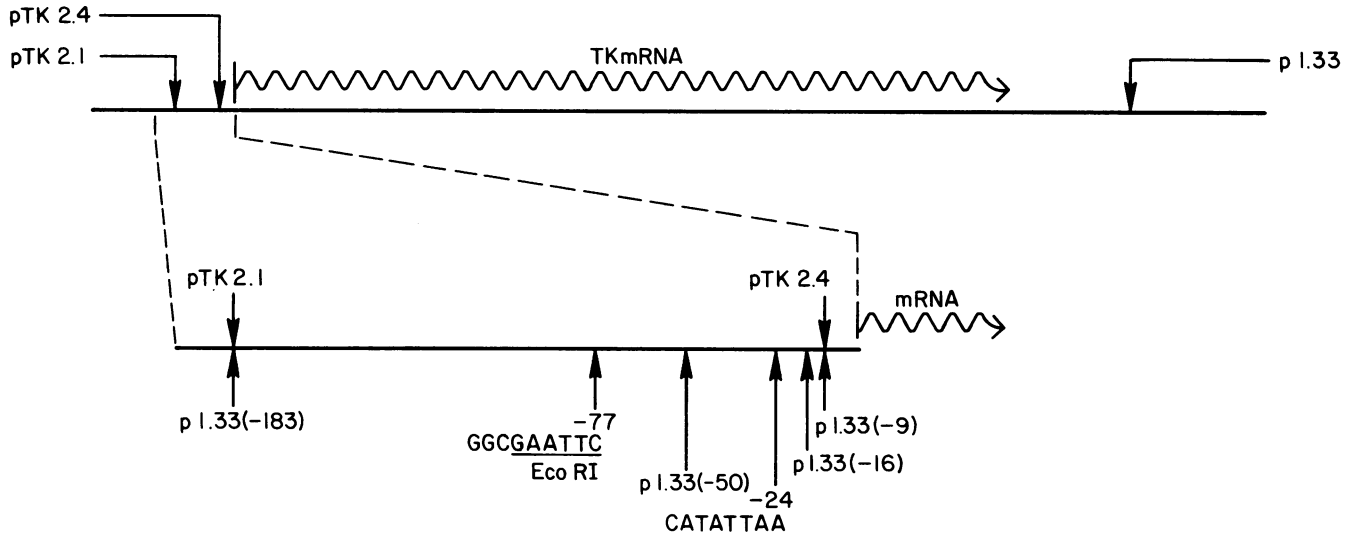

FIG. 2. Map of mutants generated *in vitro*. The top line shows the position of the three deletions on the whole 3500-base-pair HSV-1 TK insert. The bottom line is an expanded map of the 5′ untranscribed region showing the exact sites of deletion ends and insertions together with two DNA sequences thought to be important for promotion.

same as that in transformants with a single copy of an unmutated herpes TK gene. This low-level basal constitutive expression may be coming from many copies of the TK gene, each of which contributes some leakage expression, or from a copy of the TK gene which has fortuitously associated itself with an active promoter. We have not attempted to distinguish between these two possibilities. As noted above, two of the insertion mutations also have greatly lowered transformation efficiency. Transformants derived from them, with one exception (c(-9)21-5), have multiple copies of the TK gene and also have low constitutive TK levels (see Table 2b). This result suggests that these two insertion mutations have inactivated a site required for basal constitutive expression in transformants.

The Effect of Mutation on Constitutive Expression and Induction. Transformation is a complex process involving both cutting and rejoining of the input DNA molecules (29). This means that herpes TK genes will be located in a different DNA environment in each transformed cell line (30). It is even possible that mutation of the TK gene will occur as a result of the transformation process itself. These factors lead to considerable variation in the expression of the TK gene among transformants derived from a single source of DNA. To be able to detect the effects of a particular mutation on the expression of the TK gene above the noise created by this variability, several independent transformants should be analyzed. We used all of the transformants obtained that grew well enough to be tested. For each transformed line we determined the constitutive level and TK^- HSV-1-induced level of TK activity. The ratio of the activity in infected cells to that in mock-infected cells is called the induction ratio. High values of the induction ratio indicate a lot of induction of TK activity after virus infection; values of one or less indicate no induction. Table 2 contains data obtained from all transformants derived from the six mutant herpes TK genes described above, together with data from transformants derived from the parental, unmutated TK genes. Each transformed cell line in Table 2 is given a name that reflects the origin of the TK gene used to transform it. Thus, c2.0a and c2.0b are cell lines transformed with pTK2.0, whereas c2.4e and c2.4f are lines transformed with pTK2.4. Transformants derived from pTK2.4 have an average induction ratio of 0.81; this is less than 1.0 and is quite different from the average value of 2.5 observed for transformants derived from the unmutated gene in plasmid pTK2.0. This result shows that deletion of the 5′ untranscribed region of the herpes TK gene disrupts the target site for induction recognized by herpes virus. Transformants derived from the mutant plasmid pTK2.1, in which 183 base pairs of the 5′ untranscribed region of the TK gene remain, have an average induction ratio of 1.5. This value is not sufficiently different from the value obtained with the unmutated TK gene to be able to conclude that the target site for induction has been altered.

Short insertion mutants provide a more delicate probe with which to analyze the functional domains of the 5′ untranscribed region than does the large deletion present in pTK2.4. Ideally, one would want to place insertion mutations at a very large number of sites. Although we have generated insertion mutations at only four sites, several of these mutants have significant effects on expression of the herpes TK gene. Cell lines derived from p1.33(-50) have an average induction ratio less than one (Table 2b). DNA from p1.33(-50) transformed at high efficiency and gave transformants with low copy numbers of the TK gene. This insertion mutation appears to have disrupted the target site for herpes virus-mediated induction of TK without inactivating the promoter function required for expression of the basal constitutive level of TK in the transformant. An insertion mutation

Table 1. Transformation efficiencies

Exp.	Plasmid	Colonies/ plate	ng plasmid DNA/ plate	Colonies/ ng	Relative efficiency
I	pTK2.0	322	20	16.1	1.0*
	pTK2.1	143	15	9.5	0.59
	pTK2.4	173	500	0.35	0.022
II	p1.33	11	5	2.2	1.0*
	p1.33(−9)	4	1250	0.0032	0.0015
	p1.33(−183)	12	5	2.4	1.09
III	p1.33	35	5	7	1.0*
	p1.33(−50)	4	3	1.2	0.17
IV	p1.33	42	5	8.4	1.0*
	p1.33(−16)	11	1670	0.07	0.0008

The absolute transformation efficiency is given in colonies per ng of plasmid DNA.

*The relative efficiencies are given as a fraction of the parental efficiency defined as 1.0 for each experiment.

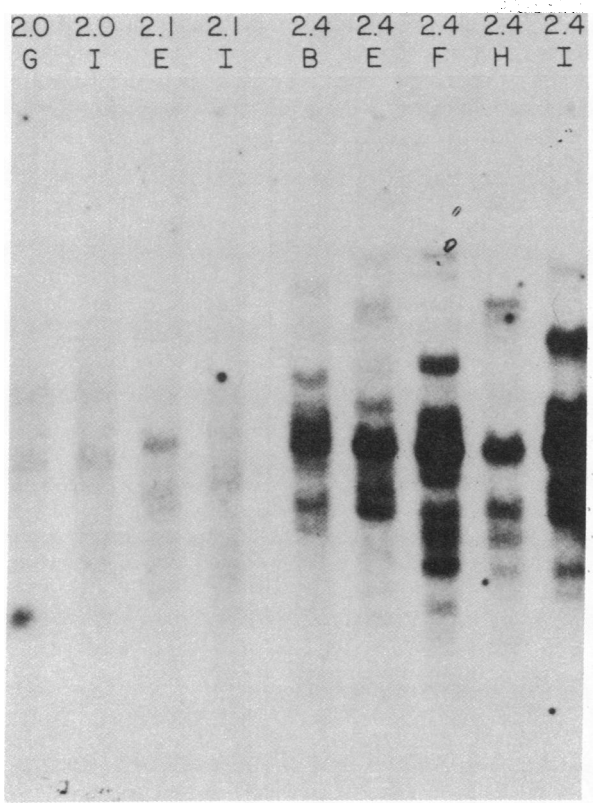

FIG. 3. Hybridization blot (28) of various transformed cell lines: DNA from cell lines that had been transformed to TK^+ with various HSV-1 TK-carrying plasmids was cut with either *Bam*HI (pTK2.0, lanes G and I) or *Bam*HI plus *Hin*dIII (pTK2.1, lanes E and I; pTK2.4, lanes B, E, F, H, and I, each letter referring to an independent transformant) and run on a 0.8% agarose horizontal gel. The DNA was transferred to nitrocellulose and hybridized with a probe of pTK2.0 DNA, labeled with ^{32}P. The same amount of cell DNA is present in each slot of the gel. The main point of this blot is to show that the deletion mutant plasmid with low transformation efficiency used at high DNA concentration produced transformants with multiple copies of plasmid DNA.

located nine base pairs upstream from the cap site has the opposite effect. Plasmid p1.33(-9) only transformed at high DNA concentrations, and most of the resultant transformed lines have many copies of the TK gene and a basal constitutive TK level below that of a transformant with a single unmutated TK gene. However, when the transformants derived from p1.33(-9) were infected with TK^- HSV-1 virus, large amounts of TK activity were produced. Indeed, the average induction ratio for all these cell lines is 16.1, a value well above that for transformants derived from the unmutated plasmid parent p1.33. This result suggests that the insertion mutation nine base pairs upstream from the cap site has inactivated a functional domain required for basal constitutive expression of the herpes TK gene resident in the transformants but has not disrupted any site required for herpes-stimulated expression of TK. The reason that such large amounts of TK are made after infection is that there are multiple copies of the TK gene in the transformant available for induction by herpes virus. DNA from p1.33(-16) was also inefficient at transformation, and most of the transformants derived from it had multiple copies of the TK gene. The value of the induction ratio for these transformants is somewhat less than that of transformants derived from the parental plasmid. This indicates an approximately parallel reduction of constitutive and induced TK levels per gene copy, suggesting that the region disrupted by the mutation in p1.33(-16) is required for both constitutive and induced expression. The fourth insertion mutation at 183 base

Table 2. Induction ratios

Plasmid	Transformant	Specific activity: Mock	Specific activity: Infected	I.F.
pTK2.0	c2.0a	4.04	12.3	3.03
	c2.0b	13.7	20.7	1.51
	c2.0d	4.47	10.5	2.36
	c2.0e	4.37	12.7	2.91
	c2.0f	5.54	15.3	2.77
	c2.0g	3.77	5.91	1.57
	c2.0h	18.6	55.9	3.0
	c2.0i	3.95	15.6	3.95
	c2.0j	12.5	20.3	1.63
	Avg =	7.88	18.8	2.52
pTK2.1	c2.1b	28.9	38.5	1.33
	c2.1g	13.1	19.2	1.46
	c2.1h	9.84	15.2	1.54
	c2.1i	4.34	7.22	1.66
	Avg =	14.0	20.0	1.50
pTK2.4	c2.4b	3.09	2.80	0.91
	c2.4e	3.36	3.05	0.91
	c2.4f	21.8	13.4	0.62
	c2.4h	3.13	2.61	0.83
	c2.4i	8.36	6.39	0.76
	Avg =	7.95	5.65	0.81
	Insertion mutants			
p1.33	c1.33b	4.34	19.1	4.39
	c1.33g	4.66	17.5	3.76
	c1.33f	4.15	10.7	2.58
	Avg =	4.38	15.8	3.58
p1.33(−183)	c(−183)1	5.19	8.89	1.71
	c(−183)2	5.67	10.6	1.86
	c(−183)6	5.61	11.1	1.98
	c(−183)7	4.44	12.5	2.82
	c(−183)9	1.86	3.58	1.93
	c(−183)10	8.29	19.2	2.31
	Avg =	5.18	11.0	2.10
p1.33(−50)	c(−50)1	5.88	3.52	0.60
	c(−50)2	5.47	5.80	1.06
	c(−50)4	4.77	6.46	1.35
	c(−50)6	5.62	3.80	0.68
	Avg =	5.44	4.90	0.92
p1.33(−16)	c(−16)2	5.34	9.93	1.86
	c(−16)4	1.67	2.14	1.28
	c(−16)5	1.15	1.47	1.28
	Avg =	2.72	4.51	1.47
p1.33(−9)	c(−9)8-1	2.68	26.0	9.68
	c(−9)8-2	2.62	20.0	7.63
	c(−9)21-2	1.10	49.7	45.1
	c(−9)21-3	0.87	20.5	23.6
	c(−9)21-4	0.90	8.1	9.0
	c(−9)21-5	1.55	2.87	1.86
	Avg =	1.62	21.2	16.1

Induction data for transformants isolated from transformations with each plasmid. The transformant names are derived from the plasmid names in an obvious manner thus c2.0a is a cell line derived from transformation with pTK2.0, c(-183)1 is derived from transformation with p1.33(-183) etc., 5 to 10 transformants of each type picked and these tables have data on all which survived and grew well enough to be tested. Induction was done as in Wilkie *et al.* (3). Assays for TK specific activity were made after 12 hr of infection and are shown as 0.01 pmol of thymidine phosphorylated per μg of protein per 15 min. I.R., induction ratio (specific activity of infected cells/specific activity of mock-infected cells).

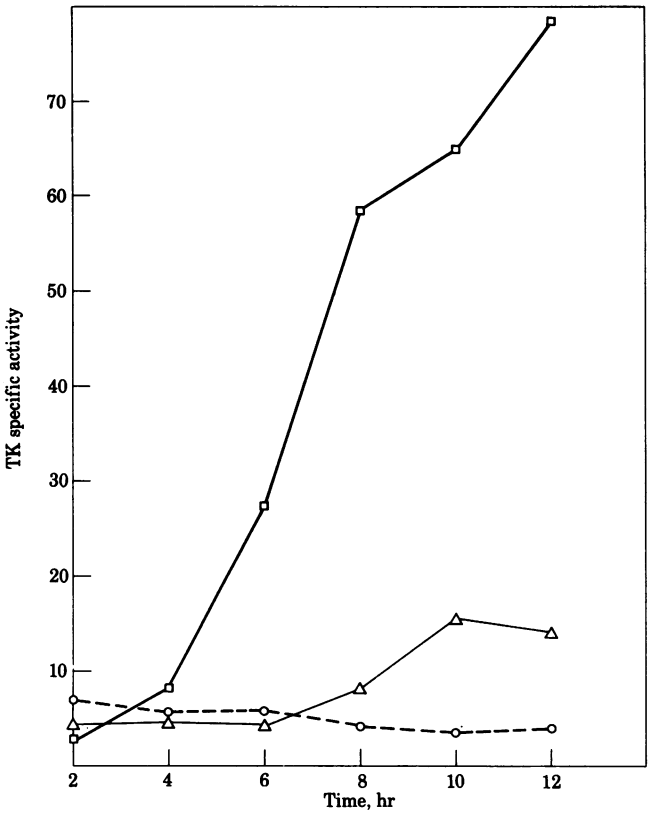

FIG. 4. Kinetics of induction of *Xho* I insertion mutants. Samples were taken every 2 hr, and the specific TK activity was determined and expressed as 0.01 pmol of thymidine phosphorylated per μg of protein per 15 min. □——□, c(-9)21-2; △——△, c.1.33b; ○----○, c(-50)1. Infection was carried out as described, with time zero being the moment of virus addition.

pairs upstream from the cap site had no effect on transformation efficiency and too small an effect on induction to be considered significant.

Kinetics of HSV-1 Induction of Transformants Carrying Mutant TK Genes. The induction ratio involves a measurement at only one point after infection. The contrast between the effects of insertion mutations at 9 and 50 base pairs upstream from the cap site is more clearly discernible in kinetic experiments which measured TK as a function of time after infection. An example is shown in Fig. 4. The level of HSV-1 TK activity eventually reached by transformant c(-9)21-2 after infection with TK^- HSV-1 is comparable to that found after infection with TK^+ HSV-1. Note that the specific activity of the HSV-1 TK in transformant c(-50)1 declines somewhat after infection, perhaps indicating that TK synthesis from existing mRNA may be inhibited after infection.

CONCLUSION AND DISCUSSION

Our analysis of the effects of mutations in the 5′ untranscribed region of the herpes TK gene leads us to the conclusion that this region controls expression in a complex manner. The experimental paradigm we used involves mutating a cloned gene *in vitro* and introducing it into cells by DNA-mediated transformation. This leads to transformants with different numbers of copies of the herpes TK gene located in different local DNA environments and probably integrated into different chromosomes. As expected, there is quite a bit of variation in both the constitutive and induced level of TK expression among transformants derived from a single mutant gene. However, unambiguous and significant effects characteristic of particular mutants are clearly discernible despite this variation. The opposite effects of linker insertion mutations 9 and 50 base pairs upstream from the cap site of mRNA enable us to distinguish between independent domains that are involved in constitutive and induced TK expression. An insertion 16 base pairs upstream from the mRNA cap site which lowers both constitutive and induced expression indicates that control of these functions overlaps to some degre.

The molecular level at which expression of the HSV-1 TK gene is being controlled cannot be determined directly from experiments of the type described here. The simplest hypothesis is that mutation of the 5′ untranscribed region is affecting mRNA synthesis. However, the initiation site of the mRNA may be affected in such a way as to interfere with its transport to the cytoplasm, its utilization by ribosomes, or its stability. It is extremely unlikely that the effects of the mutants studied here is on the structure of the thymidine kinase protein itself.

We thank M. Wigler and D. Kurtz for many useful suggestions and S. L. McKnight and W. C. Summers for giving us sequence information prior to publication, which greatly facilitated our work. Also thanks to L. Rodgers for technical help and to B. McFarland for sequence determinations. This work was supported by National Institutes of Health Grants CA09311 and GM27643 and National Science Foundation Grant PCM8003727.

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