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Identification and Mapping of Two Polypeptides Encoded Within the Herpes Simplex Virus Type 1 Thymidine Kinase Gene Sequences

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mRNA's homologous to the herpes simplex virus type 1 DNA restriction endonuclease fragment *Bam*HI *p*, which contains the thymidine kinase gene, have been identified and mapped by hybrid-arrested translation and mRNA selection. Such mRNA's, when translated in vitro, directed the synthesis of polypeptides of apparent molecular weights 43,000 (VI43) and 39,000 (VI39). mRNA for enzymatically active thymidine kinase was enriched by more than 20-fold after selection. Mapping was carried out with restriction endonuclease fragments of *Bam*HI *p*, and locations of the 5′ and 3′ termini of VI43 mRNA were deduced. Analysis of nucleotide sequences around the 5′ terminus revealed several consensus sequences commonly found at the start of eucaryotic mRNA's and which are presumably involved in initiation of transcription by RNA polymerase II. Translation of mRNA's for VI43, VI39, and the thymidine kinase enzyme was arrested only by a 1,170-base-pair region of *Bam*HI *p*. Since this region is insufficient for adjacent genes, coding sequences for VI43 and VI39 must overlap; the possible relationship of these two polypeptides is discussed. A virus-induced product equivalent to VI39 was detected in infected cells.

An important method of mapping and purifying specific mRNA's is to combine nucleic acid hybridization with in vitro translation. In such experiments, an mRNA preparation is hybridized with a purified fraction of genome DNA or RNA. Subsequent analysis involves either recovery from the hybrids and translation of selected RNAs or determination of the polypeptides whose synthesis is inhibited (termed "hybrid-arrested translation"). This approach has allowed mRNA mapping in myxoviruses (16, 20), papovaviruses (42), adenoviruses (12, 22, 23, 30, 38), and poxviruses (8). The techniques should be similarly applicable to herpesvirus mRNA's, since herpes simplex virus (HSV)-specific RNA can be translated after hybridization to viral DNA (14) and mRNA's have been enriched by hybridization to cellulose-bound DNA fragments (1, 2).

A region of the HSV type 1 (HSV-1) genome which currently commands special interest is the *Bam*HI restriction enzyme fragment *Bam*HI *p*. This fragment, located at approximately 29 to 32 map units (46), contains the information for expression of viral thymidine (pyrimidine deoxyribonucleoside) kinase (TK) when used to biochemically transform TK^- cells to a TK^+ phenotype (4, 7, 28, 29, 32, 37, 45, 47). Furthermore, cells transformed with *Bam*HI *p* increase HSV TK lvels after superinfection with TK^- virus mutants (17, 21, 47), showing that the fragment also contains sequences involved in activation by viral polypeptides (24, 35, 36). Hybrid plasmids containing *Bam*HI *p* have recently been constructed, thereby providing a source of large amounts of pure *Bam*HI *p* DNA, and detailed restriction maps have been produced (7, 10, 47). The limits of sequences necessary for biological activity have been defined by the demonstration that cleavage with *Eco*RI, *Bgl*II and *Sst*I abolish transformation, whereas a 2-kilobase pair (kbp) *Pvu*II fragment retains full activity (7, 47). Analysis of intertypic recombinants has located TK coding regions to 0.300 to 0.309 genome map units (13), and the direction of transcription of TK mRNA has also been determined (41).

With this background, studies to investigate the arrangement of mRNA coding regions within *Bam*HI *p* were initiated. Apart from its biological relevance, this fragment appeared to be suitable for such an approach because enzymatically active TK can be synthesized in vitro (9, 34) and the putative TK polypeptide, VI43, is the most abundant product upon translation of RNA extracted from cells at early times of infection (33, 35). We have been able to identify and substantially purify mRNA's homologous to *Bam*HI *p*, to map within fine limits the coding sequences

of TK mRNA, and to obtain nucleotide sequence data for the control regions around the mRNA 5′ terminus. The existence of two polypeptides, encoded by overlapping sequences within the TK gene, has also been established.

MATERIALS AND METHODS

RNA isolation. Early RNA was extracted from BHK C13 cells at 5 h after infection at 31°C with 20 PFU of HSV-1 strain 17 per cell (33). Late RNA was obtained from BS-C-1 cells at 6 h after infection at 37°C.

Plasmid DNA. A stock of *Escherichia coli* HB101 containing plasmid pTK1 (HSV-1 *Bam*HI *p* fragment inserted into pAT153 [44]) was initially provided by P. Sanders. Plasmid pTK6 was obtained by cleavage of pTK1 with *Bgl*II, followed by partial digestion with *Bam*HI. The product, consisting of pAT153 plus the small *Bgl*II-*Bam*HI fragment (see Fig. 10), was religated and transfected into *E. coli* HB101. The structure of the plasmid in the resulting ampicillin-resistant colonies was confirmed by restriction enzyme analysis, and one colony was purified and used as the source of pTK6 DNA.

Restriction endonuclease mapping of pTK1. A number of approaches, all based on recleavage of known DNA fragments, were used to refine existing maps of pTK1. (See Fig. 4, 7, and 10 for the restriction enzyme sites used to generate the DNA fragments for this study.) Fragments are described here by their approximate coordinates, in kilobase pairs, from the *Bam*HI site upstream from TK mRNA (41). The corrected positions of *Alu*I sites defining fragments A, E, and F differ from previous results (47).

Isolation of DNA from polyacrylamide gels. Gels of various polyacrylamide concentrations (5 to 10%) were cast and run in 50 mM Tris-borate (pH 8.3)–1.25 mM EDTA. After staining with ethidium bromide and visualization under long-wave UV light, the relevant bands were cut out and homogenized in 10 ml of TE buffer (20 mM Tris-hydrochloride [pH 7.5] and 2 mM EDTA). The macerated gel fragments were gently mixed for 10 to 16 h at 4°C, polyacrylamide was removed by centrifugation, and the supernatant was passed through a glass-fiber filter disk. Sodium phosphate buffer (pH 6.8) was added to 0.1 M and DNA adsorbed to hydroxylapatite. After thorough washing at 60°C with 0.2 M sodium phosphate buffer, DNA was eluted with 0.5 M sodium phosphate buffer. *E. coli* rRNA (20 μg) was added, and the eluate was dialyzed against TNE buffer (TE buffer plus 0.1 M NaCl) and precipitated in ethanol.

Portions of isolated DNA fragments were electrophoresed, and negatives of the UV-illuminated, ethidium bromide-stained bands were prepared. The negatives were scanned, using a Joyce-Loebl microdensitometer, to assess the purity and concentrations of DNA fragments. None of the fragments used in the experiments reported here contained detectable contamination by others.

DNA sequencing. DNA sequences around the 5′ terminus of the TK gene were determined by using the chemical degradation system of Maxam and Gilbert (26, 27), and also by cloning fragments into phage M13 derived vectors (11) and copying the recombinant single-stranded phage DNA in the presence of specific chain terminators (39, 40; F. Sanger, A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe, J. Mol. Biol., in press).

For the chemical degradation system, pTK1 DNA was cleaved with *Eco*RI or with *Bgl*II, labeled at the 5′ termini with polynucleotide kinase and [γ-^{32}P]ATP, and then cleaved with *Hin*fI. The resulting single end-labeled fragments were separated by electrophoresis in polyacrylamide gels, and the appropriate bands were recovered. All sequencing gels contained 9 M urea.

*Eco*RI-cleaved fragments of pTK1 DNA were cloned into the *Eco*RI site of the phage vector M13mp2*am* and propagated in *E. coli* JM101 (11). Sequencing was carried out as described by Sanger et al. (J. Mol. Biol., in press) with the short universal primer of Anderson et al. (3).

Hybrid arrest. Plasmid DNAs were cleaved with *Eco*RI, extracted with phenol, and precipitated with ethanol. Two micrograms of restricted plasmid DNA, or the molar equivalent amount of a particular isolated fragment, was denatured in 85 μl of 95% formamide (deionized) containing 60 mM PIPES [piperazine-*N*,*N*-bis(2-ethanesulfonic acid)] (pH 7.4) at 95°C for 5 min. HSV-1-infected cell cytoplasmic RNA (6 μl; 8 mg/ml) was added, followed by addition of 8 μl of 5 M NaCl, and the hybridization mixture was rapidly transferred to a 58°C bath. After incubation for 1 h, 1 ml of HSB (500 mM KCl; 20 mM Tris-hydrochloride [pH 7.5]; 1 mM EDTA) was added. Polyadenylic acid-containing RNA was selected on a small oligodeoxythymidylic acid-cellulose column and precipitated with ethanol after addition of *E. coli* rRNA. Pelleted RNA was dried, dissolved in deionized water, and used for in vitro translation either directly or after denaturation by heating at 95°C for 2 min.

mRNA selection. The technique of Ricciardi et al. (38) was modified for selection of pTK1-specific mRNA's. A nitrocellulose filter (13-mm diameter; Schleicher and Schuell, Dassel, West Germany), loaded with 100 μg of plasmid DNA, was cut into small pieces and incubated for 2 h at 55°C with early or late cytoplasmic RNA, dissolved in hybridization buffer (40% formamide [deionized], 600 mM NaCl, 50 mM PIPES [pH 7.4], and 1 mM EDTA). The hybridization mixture was then removed and precipitated after addition of 1 volume of water and 2.5 volumes of ethanol to yield the nonhybridized fraction. The filter pieces were incubated at 55°C for 10 min in hybridization buffer, then washed 10 times at 60°C with SSC (0.15 M NaCl and 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate, followed by 2 washes at 60°C with SSC containing 2 mM EDTA. The pieces were then washed for two 5-min periods in SSC–2 mM EDTA and once with cold deionized water. A 100-μl amount of 95% formamide–50 mM PIPES (pH 7.4) was added, and incubation was continued at 60°C for 5 min. The formamide fraction was stored, and the filter pieces were incubated with 300 μl of deionized water for 2 min at 95°C. This fraction was combined with the 95% formamide fraction, *E. coli* rRNA (15 μg) and NaCl (0.2 M) were added, and RNA was precipitated after addition of 2.5 volumes of ethanol

(selected RNA fraction). The RNA was washed with ethanol, dried, dissolved in water, and used for in vitro translation. In some experiments, the selected RNA fraction was repurified on a small oligodeoxythymidylic acid-cellulose column, as described above.

In vitro protein synthesis. RNA samples were translated in a micrococcal nuclease-treated fractionated reticulocyte system (31, 33, 35). In vitro-synthesized TK enzyme was assayed as described previously (34).

Labeling of infected cell polypeptides. BHK cells were pulse-labeled at 37°C for 15 min in phosphate-buffered saline containing 500 μCi of [^{35}S]methionine (The Radiochemical Centre, Amersham, England) per ml. Cell monolayers were then washed with ice-cold phosphate-buffered saline, harvested, and prepared for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25).

Polypeptide nomenclature. As described previously (25, 33) polypeptides synthesized in vitro and in vivo are classified according to their apparent weights in thousands and are prefixed with VI or Vmw, respectively.

Biological containment. All manipulations with bacteria containing plasmid pTK1 or pTK6 or phage M13mp2*am* were conducted in a category II laboratory, as recommended by GMAG.

RESULTS

Selection of mRNA's which hybridize to pTK1 DNA. mRNA's were selected by incubation of late RNA from infected BS-C-1 cells with nitrocellulose filters loaded with plasmid DNA. After the filters were thoroughly washed, hybridized RNA was eluted and translated. Fig. 1 shows that this procedure selected mRNA's coding for a major (VI43) and minor (VI39) species from the array of late virus-induced polypeptides. These mRNA's were not selected from infected cell RNA by pAT153 DNA filters or from uninfected cell RNA by pTK1 DNA, in which cases only endogenous reticulocyte bands were observed. The specificity of the procedure was further demonstrated by the facts that VI43 was not the most abundantly represented species in translation products of the original RNA and VI39 was undetetable before mRNA selection.

To investigate whether mRNA for active TK enzyme was also enriched by hybridization to pTK1 DNA, RNA from BHK cells at 5 h after infection at 31°C, a time when TK mRNA levels are maximal, was used. Selected and nonhybridized RNAs were examined for the ability to stimulate incorporation of ^{14}C-amino acid or to direct synthesis of TK. Fig. 2 (A and B) shows that selected RNA was less active in stimulating protein synthesis, but had marginally greater TK mRNA content. The measured enrichment of TK mRNA (TK activity per unit of amino acid incorporation of selected RNA compared

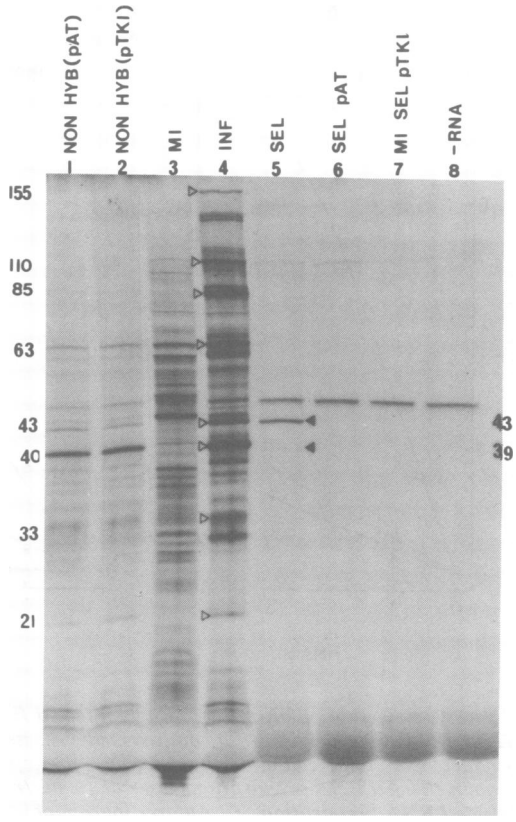

FIG. 1. *Selection of pTK1-specific mRNA. Mock-infected (MI) or late infected (INF) BS-C-1 cell RNA was hybridized to filters containing pTK1 DNA or pAT153 DNA. Both nonselected and selected mRNA preparations were translated in vitro. RNA preparations used were the following: INF cell RNA not selected by pAT153 DNA (track 1), INF cell RNA not selected by pTK1 DNA (track 2), original MI BS-C-1 cell RNA (track 3), original INF cell RNA (track 4), INF cell RNA selected (SEL) by pTK1 DNA (track 5), INF cell RNA selected by pAT153 DNA (track 6), MI cell RNA selected by pTK1 DNA (track 7), and no added RNA (track 8). For reference, the molecular weights (in thousands) of some prominent virus-induced polypeptides present are shown in track 4 (◁). Selected polypeptides are labeled in track 5 (◀). A more thorough categorization of late virus-induced polypeptides synthesized in vitro is given elsewhere (32, 34).*

with nonhybridized RNA) varied from 20- to 50-fold in repeated experiments. An accurate value is difficult to obtain because the stimulation of ^{14}C-amino acid incorporation by selected RNA was so small.

Translation of relevant RNA fractions (Fig. 2C) revealed that the polypeptides directed by selected mRNA were VI43 and VI39, as found with late RNA (Fig. 1). The greater abundance of VI43 and VI39 mRNA was reflected in a

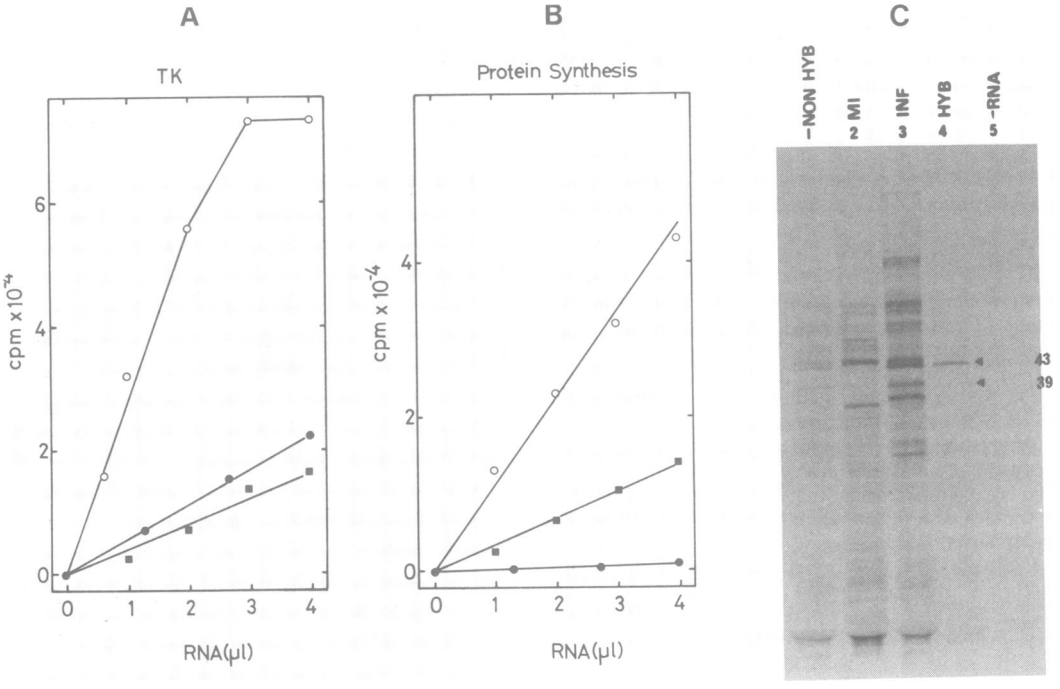

FIG. 2. *Selection of TK mRNA. Early infected (INF) BHK cell RNA was hybridized to filters containing pTK1 DNA. The original* (○), *nonselected* (■), *and selected* (●) *fractions were translated in vitro, and products were analyzed either by assay of TK enzyme (A) or incorporation of [^{14}C]amino acid mixture into protein (B). All RNA preparations were at 1.2 mg/ml, this being achieved by addition of E. coli rRNA to the selected RNA preparation. (C) Autoradiogram of [^{35}S]methionine-labeled polypeptides directed by the three RNA preparations, together with mock-infected (MI) BHK cell RNA and no added RNA.*

greater yield of selected mRNA, as indicated by the higher intensities of these polypeptides, as compared with the major endogenous band (molecular weight, ca. 50,000).

The selection experiments, therefore, show that the major mRNA encoded by *Bam*HI *p* at both early and late times of infection specifies VI43. Another polypeptide, VI39, was also synthesized, and TK mRNA was enriched, strongly suggesting that TK activity resides in either VI43 or VI39.

Hybrid-arrested translation with pTK1 DNA. An alternative way of mapping mRNA's is by hybrid-arrested translation (16, 30). This method can be used with small DNA fragments and might give more precise locations than mRNA selection, as it has been reported that DNA fragments shorter than 300 bp do not bind reproducibly to nitrocellulose filters (38). Figure 3 (left) shows translation of early RNA after incubation with pTK1 or pAT153 DNA under conditions which favor annealing of RNA to DNA. With both plasmids, three polypeptide bands (VI136, VI129, and VI88) disappeared after hybridization, and one (VI85) was synthesized in reduced amounts. These returned, to some extent, after denaturation of hybrids, but clearly their arrest cannot be attributed to *Bam*HI *p* sequences. The effect is presumably due to some unusual structural features of the mRNA's, since it can also be obtained by mock-hybridization without addition of DNA (data not shown).

Hybridization with pTK1 DNA, as compared with that of pAT153 DNA, additionally prevented synthesis of VI43 and VI39. mRNA's for these polypeptides regained activity upon denaturation of hybrids, thus demonstrating that they are encoded by *Bam*HI *p*. Figure 3 (right) shows that the two arrested bands correspond to selected VI43 and VI39. It should be noted, however, that VI39 comigrates with a minor cell polypeptide, and that RNA preparations containing high levels of its mRNA (i.e., early RNA) must be used for efficient detection in hybrid-arrest experiments.

Analysis of sequences at the 5' terminus of TK mRNA. Initial experiments were aimed at locating those *Bam*HI *p* sequences which are represented in the mRNA's for VI43 and VI39. Fragments of pTK1 generated by *Sma*I or *Alu*I were used in hybrid-arrested translation, and

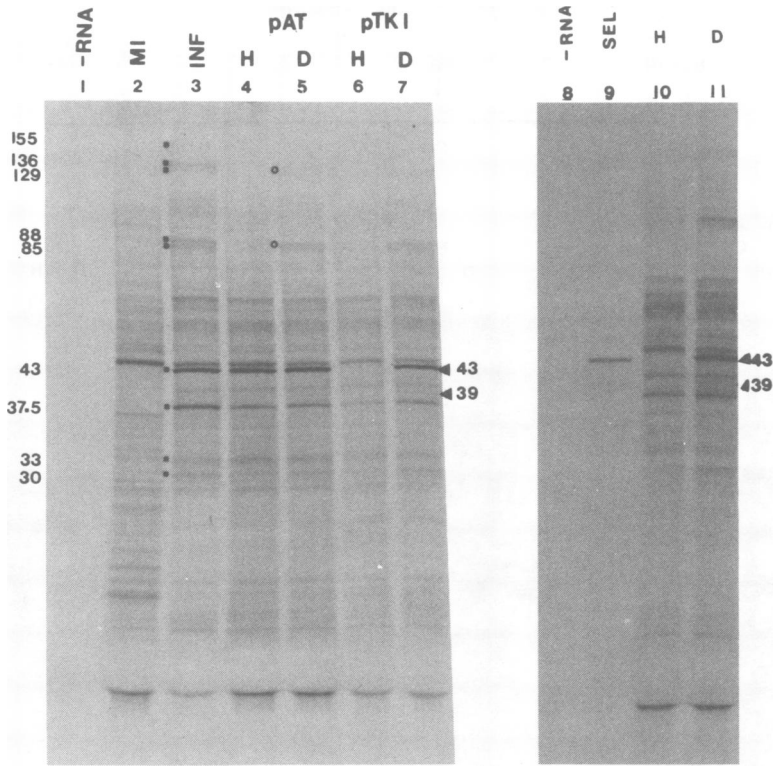

FIG. 3. *Hybrid arrest of translation by pTK1 DNA. Early infected (INF) BHK cell RNA was hybridized to pAT153 DNA or pTK1 DNA and directly translated (tracks labeled H) or denatured before translation (tracks labeled D). RNA preparation used were no added RNA (tracks 1 and 8), mock-infected cell RNA (track 2), early INF cell RNA (track 3), hybridized to pAT153 DNA (track 4), track 4 sample denatured (track 5), hybridized to pTK1 DNA (track 6), track 6 sample denatured (track 7). From a separate experiment, the products of pTK1-selected RNA (track 9) were compared (tracks 8 through 11) with those of pTK1-arrested RNA (track 10). (Track 11 shows track 10 sample denatured.) Symbols: ■, known virus-induced polypeptides;* (○) *polypeptides whose synthesis is arrested by incubation with either pAT153 or pTK1 DNA.*

these experiments showed that such sequences lay within the 2.02-kbp *Pvu*II fragment (0.50 to 2.52 kbp from the 5′ *Bam*HI site) previously reported to possess full transforming activity (7) (data not shown). Further definition was achieved by use of small DNA fragments generated by double digests or by recleavage of larger fragments followed by a second cycle of electrophoresis and purification. Figure 4 shows hybrid-arrested translation in the region which should contain the mRNA 5′ terminus and untranslated sequences of the TK gene (42). All five *Bst*NI fragments, derived by cleavage of the pTK1 *Bgl*II-*Sst*I fragment (0.74 to 1.24), arrested translation of VI43 and VI39. Fragments C (−0.38 to 0.61) and D (0.61 to 0.74) from the *Eco*RI-*Bgl*II double digest failed to arrest translation under standard conditions, whereas fragment B (0.74 to 3.0) of the same digest was fully active. This experiment, therefore, shows that a fragment as small as 78 bp (the estimated size of *Bgl*II-*Sst*I-*Bst*NI fragment E) can efficiently arrest translation.

Inspection of Fig. 4, tracks 7 through 16, shows a minor polypeptide of an apparent molecular weight of 60,000 whose synthesis was arrested by all *Bgl*II-*Sst*I-*Bst*NI fragments. This species has not been resolved so clearly in any other autoradiograms, and it is therefore not possible to determine whether the effect is due to *Bam*HI *p* DNA or to the self-arrest described above.

Nucleotide sequences of the potentially interesting regions around the *Bgl*II site were determined by the Maxam-Gilbert method (26) and by the use of dideoxytriphosphates in copying single-stranded fragments cloned in phage M13mp2. The 440-bp region depicted in Fig. 5 combines data from fragments end-labeled at the *Eco*RI site or the *Bgl*II site and from *Eco*RI fragments cloned in M13mp2.

Five ATG codons, potential protein synthesis initiators, can be detected. The first four of

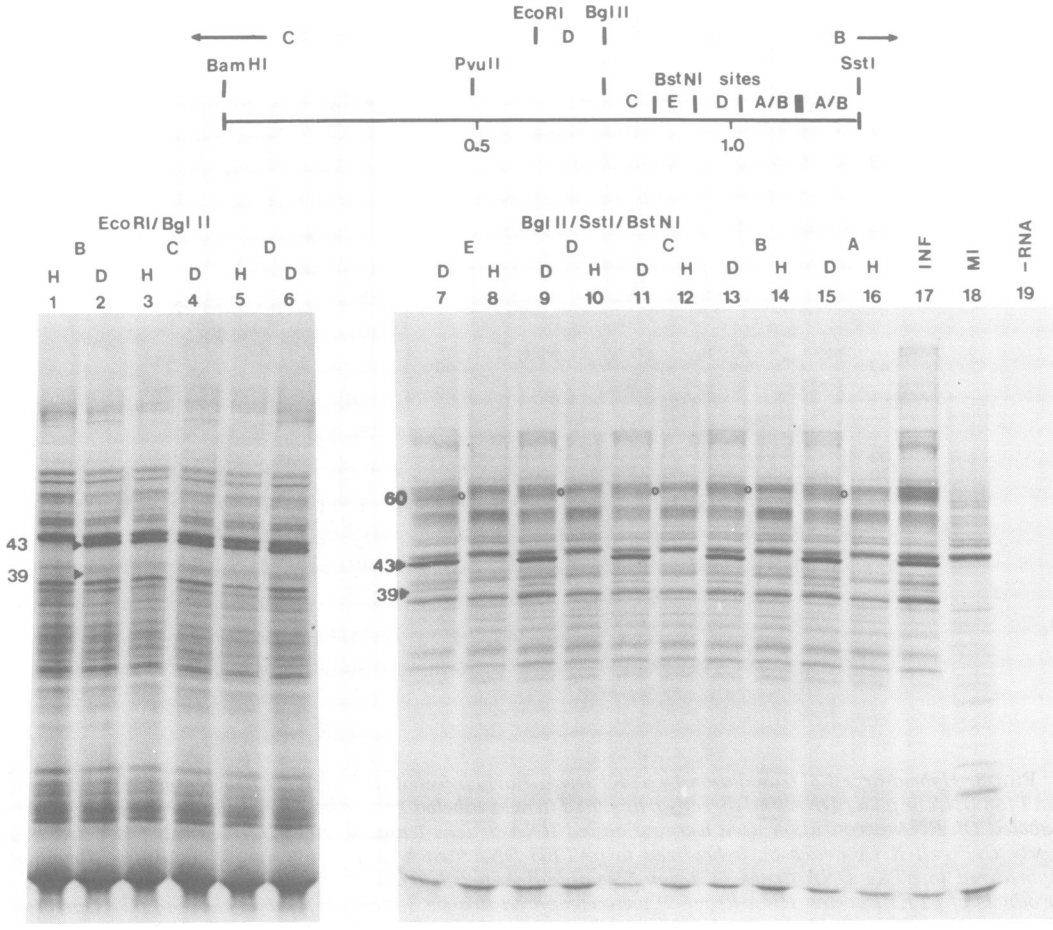

FIG. 4. *Hybrid-arrested translation with TK mRNA 5′ sequences. Fragments of pTK1 from an EcoRI-BglII double digest (tracks 1 through 6) or a BstNI digest of the BglII-SstI fragment (tracks 7 through 16) were used. RNA was translated directly (H) or after denaturation (D). The origins of the fragments are shown in the map (BglII-SstI-BstNI fragments A and B have not been ordered). Track 17, translation products of the original RNA; track 18, BHK cell RNA; and track 19, endogenous products of the cell-free system. Symbols: ○, an additional polypeptide (60) which is arrested by all BglII-SstI-BstNI fragments.*

these, all upstream from the *Bgl*II site, are in closed reading frames since protein synthesis termination codons follow closely in the same phase, whereas the fifth, located 57-bp downstream from the *Bgl*II site, is in an open frame as far as the analysis can detect. Provided splicing does not occur in the region analyzed, this is the only candidate site for protein synthesis initiation.

Upstream from the *Bgl*II site, various sequences previously recognized in the initiation regions of many eucaryotic genes can be identified (5, 6). Most striking is the AT-rich region, which is very similar to a Hogness box (GTATAATAG), at positions 197 through 207. This sequence is usually approximately 30 bp upstream from an mRNA 5′ terminus, and the sequence CGAATAC (224 through 230) in the appropriate position resembles the consensus sequences found at the mRNA 5′ capping site (6). At 5 and 12 bp upstream from the Hogness box are two GGTCC sequence, which are very similar to the GATCC consensus (6). Two other regions further upstream from the mRNA terminus are at positions 144 through 152 (the −70 to −80 homology) and at positions 83 through 92, an AT-rich sequence commonly found 130 to 150 bp upstream from the mRNA terminus (5).

Consideration of the organization of sequences therefore suggests that an mRNA is initiated approximately 50 bp upstream from the *Bgl*II site, a conclusion which is compatible with initiation of protein synthesis 57 bp downstream from the *Bgl*II site.

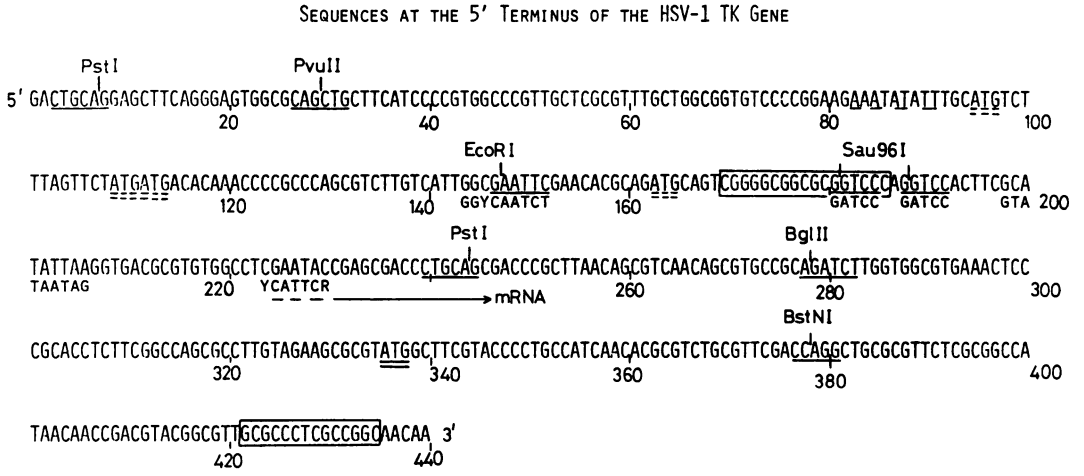

FIG. 5. *Sequence of 440 bp at the 5' terminus of the TK gene. Previously recognized consensus sequences are shown at appropriate positions, apart from the −140 AT-rich region, which has a broken underline. The proposed initiation site for protein synthesis has a solid double underline; other ATG codons (all in a different, closed reading frame) have broken double underlines. Restriction sites are given solid underlines, and two high GC regions which may be important in interpreting hybrid-arrested translation are boxed. Y, Pyrimidine; R, purine.*

In view of the implications from study of the TK gene 5' sequences, the effects of the region upstream from the *Bgl*II site on mRNA selection and hybrid-arrested translation was investigated further, since no arrest could be obtained by *Eco*RI-*Bgl*II fragments from this region (Fig. 4). To facilitate these experiments, plasmid pTK6, which contains only the small *Bam*HI-*Bgl*II fragment of pTK1 (0.0 to 0.74), was constructed (see Fig. 10). This plasmid DNA was able to select mRNA for VI43 and VI39 apparently as efficiently as pTK1 DNA (Fig. 6, tracks 1 through 6), showing that sequences of these mRNA's extend upstream from the *Bgl*II site. When hybrid-arrested translation was investigated further, it was found that reduction in hybridization temperature to 54°C and a fivefold increase in equivalent DNA concentration allowed detectable arrest of VI43 and VI39 (Fig. 6, tracks 7 through 14). Presumably the requirement for lower temperature reflects the lower GC content of the region immediately upstream from the *Bgl*II site. A further interesting point is that translation was arrested more efficiently when *Sau*96I-cleaved pTK6 DNA, as opposed to *Eco*RI-cleaved DNA, was used. The enzyme *Sau*96I cleaves adjacent to a very high GC region that is 17 to 34 bp downstream from the *Eco*RI site. This region should reassociate strongly under the hybridization conditions, and may reduce the availability of downstream sequences for hybridization with mRNA.

The conclusion from these experiments is that VI43 and VI39 mRNA sequences extending from the *Bgl*II site can be detected by RNA selection and hybrid-arrested translation under appropriate conditions. The altered characteristics of hybrid-arrested translation suggest that the mRNA sequences do not extend entirely through the *Eco*RI-*Bgl*II fragment D (0.61 to 0.74), since *Bgl*II-*Sst*I-*Bst*NI fragment E, a fragment with similar overall nucleotide composition including a region of very high GC, inhibited translation under standard conditions (Fig. 4). It is most likely that the mRNA 5' terminus is 20 to 30 bp downstream from the Hogness box and that the protein synthesis initiation codon is 57 bp downstream from the *Bgl*II site. However, from the results presented here, the possibility that sequences between the *Pvu*II site and the very high GC region (0.50 to 0.63) specify a short 5' leader, which is spliced onto 5' noncoding sequences, cannot be excluded.

Sequences at TK mRNA 3' terminus. Hybrid-arrested translation with fragments derived from the 3' end of the TK gene is shown in Fig. 7. Three fragments within *Sma*I fragment A (0.25 to 1.91) inhibited synthesis of VI43 and VI39. Additionally, truncated polypeptides, presumably resulting from ribosome termination at the duplexed site, could be detected, and these are labeled in tracks 2, 4 and 6 of Fig. 7. The intensities of these bands, as compared to those of VI43 after denaturation, were lower than expected and insufficient for confident alignment of restriction sites with coding sequences. The 16,000-molecular-weight species formed a novel band, but the 29,000- and 35,000-molecular-

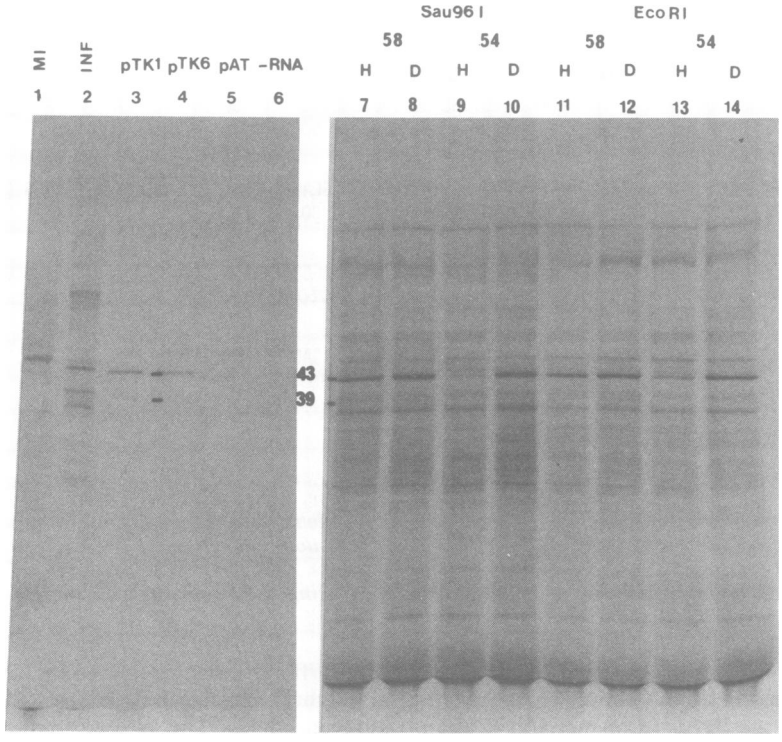

FIG. 6. *RNA selection and hybrid-arrested translation with pTK6 DNA. Shown are translation products of mRNA's selected by pTK1, pTK6, and pAT153 DNA filters (tracks 3, 4, and 5, respectively); products of mock-infected (MI) BHK cell RNA (track 1), infected (INF) cell RNA (track 2), endogenous proteins (track 6); and hybrid-arrested translation with pTK6 DNA, cleaved with Sau96I (tracks 7 through 10) or EcoRI (tracks 11 through 14), at 54°C or 58°C. RNA was translated directly (H) or after denaturation (D).*

weight bands were only detectable as enhancements of other polypeptides. A product of molecular weight 35,000 has been produced by arrest of pTK1-selected mRNA with *Sma*I-*Hin*fI-*Ava*I fragment A (1.68 to 1.91) (data not shown), and therefore appears to be a genuine truncated polypeptide.

The *Sma*I-*Alu*I-*Dde*I fragment B (2.31 to 2.55) did not arrest translation of VI43 and VI39, but fragmet A from this digest (1.91 to 2.31) partially inhibited synthesis of both polypeptides (compare the intensities of actin and VI43 in Fig. 7, track 8). This curious effect could be a real property of sequences in *Sma*I-*Alu*I-*Dde*I fragment A, or could be due to contamination by degradation products of *Sma*I fragment A (0.25 to 1.91) during electrophoresis. Hybrid-arrested translation was therefore carried out with increasing concentrations of a *Bgl*II-SstI fragment (0.74 to 1.24), *Sma*I fragment B (1.91 to 2.69) isolated from a *Sma*I digest of pTK1, or *Sma*I fragment B, isolated from a *Sma*I-*Bgl*II-*Sst*I-*Hin*fI digest of pTK1, which reduced *Sma*I fragment A to fragments smaller than *Sma*I fragment B and should eliminate any possible contamination. Figure 8 shows that maximum inhibition of VI43 synthesis was achieved with approximately 1 μg equivalent (i.e., derived from 1 μg of pTK1 DNA) of each fragment, and that increasing the concentration of *Sma*I fragment B a further sixfold did not give a greater effect. Similar results were obtained with whichever digest was used to generate *Sma*I fragment B, and, therefore, the partial arrest is a characteristic of this fragment.

The size of the 35,000-molecular-weight truncated polypeptide produced by arrest with *Sma*I-*Hin*fI-*Ava*I fragment A suggests that the coding sequences of VI43 end at approximately 230 bp downstream, i.e., very close to the *Sma*I site, provided there are no introns in this region. Partial arrest with *Sma*I fragment B might therefore result from hybridization with the 3′ untranslated region or from an overlap into coding sequences producing, at reduced efficiency, a truncated polypeptide whose reduced molecular weight could not be detected. Since it has been shown that hybridization of plasmid DNA to the entire 3′ noncoding region of β-globin mRNA does not affect translation in vitro (19),

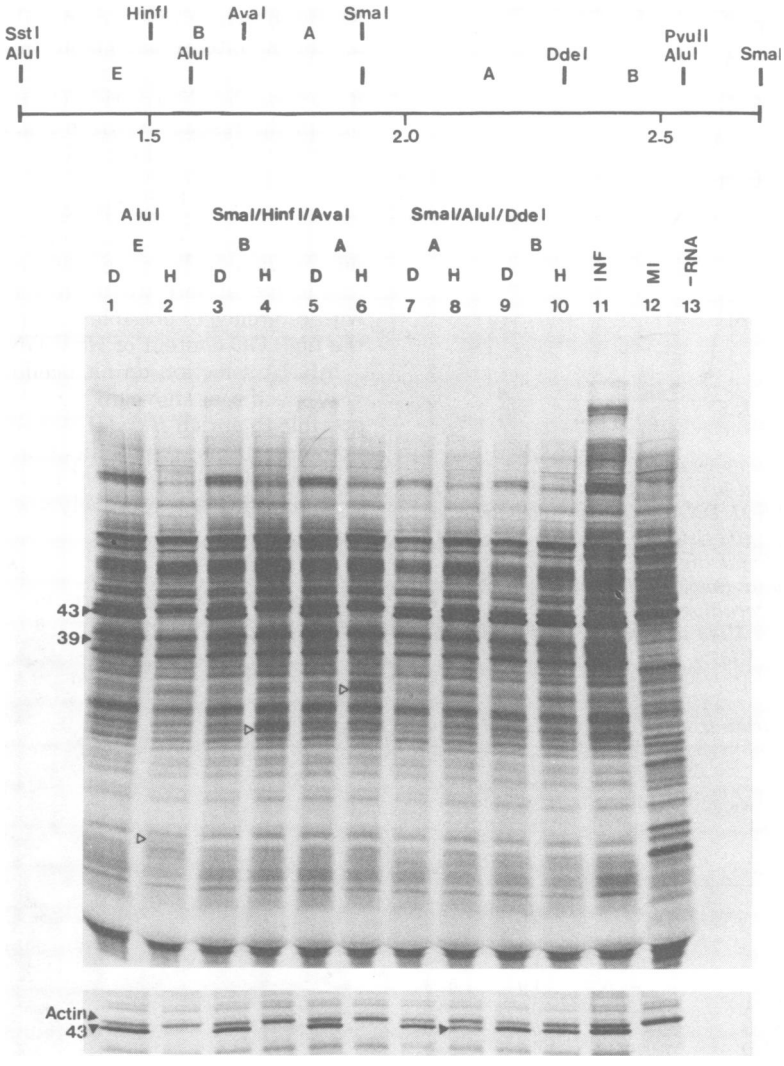

FIG. 7. *Hybrid-arrested translation with 3' fragments of TK gene sequences. Five fragments of pTK1 DNA, indicated in the map, were used in hybrid-arrested translation (tracks 1 through 10). RNA was either translated directly (H) or after denaturation (D). Shown are translation products of infected (INF) cell RNA (track 11), mock-infected (MI) BHK cell RNA (track 12) and endogenous proteins (track 13). The shorter exposure on the lower panel demonstrates partial inhibition of VI43 synthesis by SmaI-AluI-DdeI fragment A more clearly (track 8). Symbols: ◁, putative truncated polypeptides.*

the latter interpretation appears most likely. The 3' limit of coding sequences for VI43 and VI39 therefore probably lies within *Sma*I-*Alu*I-*Dde*I fragment A, no more than 30 bp from the *Sma*I site.

Hybrid-arrest of TK synthesis. The coding sequences for TK enzyme were also analyzed by hybrid-arrested translation, and Table 1 shows that fragments which inhibited synthesis of TK mapped in the same region as VI43 and VI39. It is interesting to note that the putative truncated polypeptide generated by *Sma*I fragment B retained TK activity.

Synthesis in vivo of a polypeptide equivalent to VI39. Coding sequences for VI43 and VI39 lie within the same 1,170 bp between the *Bgl*II (0.74) and *Sma*I (1.91) sites and presumably have the same polarity, since they behave in the same aberrant way to hybrid arrest with fragments from the mRNA termini. Since there is insufficient coding capacity for two adjacent polypeptides of these sizes, their sequences must overlap. It was considered important to determine whether both polypeptides were synthe-

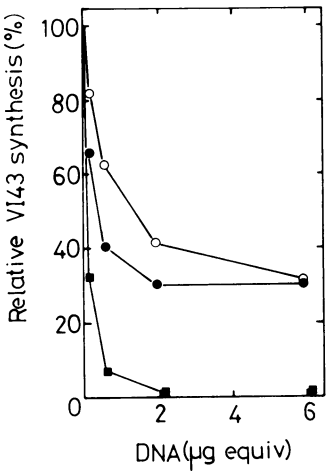

FIG. 8. *Hybrid-arrest of VI43 with SmaI fragment B and BglII-SstI DNA. Various concentrations of fragments of BglII-SstI (■), SmaI fragment B isolated from a SmaI digest (○), or SmaI fragment B isolated from a SmaI-BglII-SstI-HinfI digest (●) were used. Autoradiograms were scanned, and the radioactivity in VI43 was compared with neighboring, unaffected VI37.5.*

TABLE 1. *Hybrid-arrest of TK synthesized in vitro*

DNA fragment used in hybrid-arrest	Location (kbp)[a]	[^{3}H]thymidine phosphorylated (cpm × 10^3)	
		Hybrid-ized	Dena-tured
pTK1		0.0	72.4
pAT153		78.9	59.4
*Sma*I-B	1.91–2.69	15.3	83.8
*Hin*fI-*Sma*I	1.50–1.91	1.1	91.9
*Alu*I-E	1.24–1.58	1.0	52.3
*Bgl*II-*Sst*I-*Bst*NI-A	1.01–1.24	0.0	68.2
*Bgl*II-*Sst*I-*Bst*NI-B		0.0	67.6
*Bgl*II-*Sst*I-*Bst*NI-D	0.92–1.01	0.5	58.0
*Bgl*II-*Sst*I-*Bst*NI-C	0.82–0.92	0.0	66.4
*Bgl*II-*Sst*I-*Bst*NI-E	0.74–0.82	0.0	48.8
pTK6, 54°C[b]	0.0–0.74	5.0	72.6
pTK6, 58°C[b]	0.0–0.74	53.6	72.4
*Eco*RI-*Bgl*II-C	−0.38–0.61	52.0	47.2

[a] Location of fragments is given in kbp downstream from the *Bam*HI site.

[b] Hybrid-arrest with pTK6 used Sau96I-cleaved DNA at fivefold higher concentration at the temperature indicated.

sized in infected cells as well as in vitro. Figure 9 shows pulse-labeled polypeptides from BHK cells infected with wild-type HSV-1 or the TK$^-$ mutant dPyK$_1^{-7}$, which fails to synthesize VI43 (33). Comparison of tracks 4, 5, 6, and 7 of Fig. 9 reveals that a band comigrating with VI39 and of approximately the same relative intensity was detected in wild-type HSV-1-infected cells, but not in mock-infected or dPyK$_1^{-7}$-infected cells. The probable reason why the VI39 equivalent has not previously been detected in infected cells is that the intense band below it is rapidly processed to a slower migrating form which obscures it. A short labeling period (15 min was used to obtain the results shown in Fig. 9) is necessary to prevent this masking effect.

DISCUSSION

Many of the techniques described here have not previously been applied to herpesviruses, and it is therefore worthwhile to discuss them with reference to possible problems arising from the high GC content of HSV DNA.

mRNA selection on nitrocellulose filters was very specific, as shown in Fig. 1 through 3. It was possible to enrich *Bam*HI *p*-specific RNAs with no detectable contamination from other mRNA's. The efficiency of hybridization to filter-bound DNA is relatively low, with only 5 to

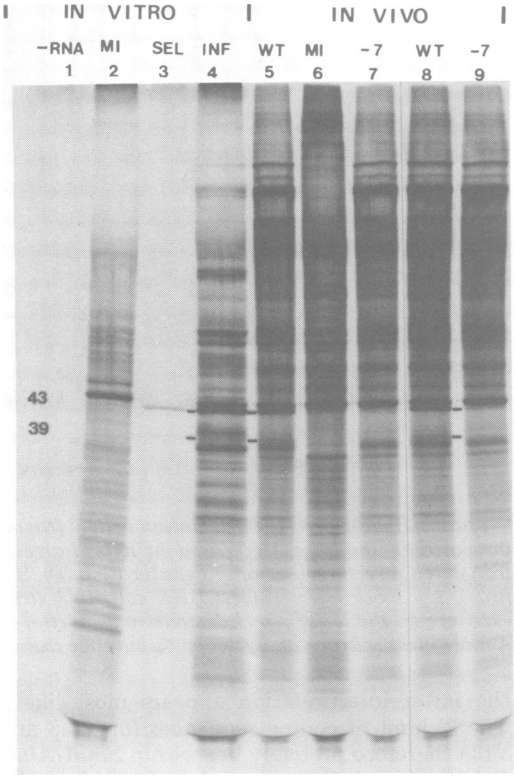

FIG. 9. *Synthesis of VI43 and VI39 in vitro and in vivo. Shown are endogenous products of the translation system (track 1), polypeptides directed by mock-infected (MI) BHK cell RNA, pTK1-selected (SEL) RNA, and infected (INF) BHK cell RNA (tracks 2 through 4, respectively), polypeptides synthesized in BHK cells at 5 h after infection at 31°C with wild-type (WT) HSV-1 or dPyK$_1^{-7}$ (tracks 5, 7, 8, and 9), or MI BHK cells (track 6).*

10% of the original mRNA activity recovered, and high RNA concentrations are therefore required. The high GC content does not seriously affect hybridization of RNA to denatured, immobilized pTK1 DNA, and the finding that pTK6 DNA selects mRNA shows that only a small region of sequence homology is necessary. The washing procedure described for adenovirus mRNA's (39) removes nonspecifically bound HSV mRNA's.

Hybrid-arrested translation is a potentially more sensitive method of mRNA mapping and the studies presented here have used the smallest fragments yet reported. Since both DNA-DNA and DNA-RNA interactions can occur, temperature control to favor DNA-RNA reactions must compensate for GC content (15, 43). This results in the use of relatively high hybridization temperatures for HSV mRNA's. Hybrid-arrested translation could not detect mRNA's for minor polypeptides which comigrate with major cellular or viral bands, but otherwise the specificity seems equal to that of mRNA selection. Some mRNA's, however, cannot be mapped since they are inactivated by mock-hybridization, emphasizing the importance of including appropriate controls. DNA fragments as small as 78 bp efficiently arrest translation, provided they hybridize entirely to mRNA coding regions. When the area of overlap includes mRNA ends, the results become more difficult to interpret and further investigation is necessary. Production of truncated polypeptides was less efficient than recently described for influenza mRNA's in the wheat germ system (20). Possibly, ribosome release at the abnormal site is inefficient, and the resulting decreased synthesis of truncated products is more marked in the reticulocyte lysate, as it supports more rounds of translation than the wheat germ system.

Hybrid-arrest and selection experiments with pTK6 DNA suggest that sequences represented in VI43 and VI39 mRNA end within 90 bp (the site of the very high GC region) upstream from the *Bgl*II site. Analysis of the nucleotide sequence in this region shows many features characteristic of eucaryotic mRNA 5′ termini. The positions of the consensus sequences found in other genes imply that an mRNA initiation site exists at approximately 50 bp from the *Bgl*II site. Recently, S1 nuclease mapping has identified a major mRNA 5′ terminus at this position (48), reinforcing the conclusions made here. If the AT-rich region 80 bp further upstream is necessary for initiation of transcription, the failure of *Eco*RI-cleaved pTK1 DNA to transform TK^- cells can readily be interpreted.

Location of the 3′ limit of coding sequences close to the *Sma*I site (1.91) is based on the properties of hybrid-arrest with *Sma*I fragment B and the sizes of truncated polypeptides generated by fragments hybridized to the 3′ end of VI43 mRNA. The finding that the putative shortened polypeptide produced by hybridization with *Sma*I fragment B has TK activity might explain the ability of *Sma*I-cleaved plasmids to transform cells (7), and it would be interesting to examine TK mRNA in such cells.

The experiments reported here have derived a precise location of coding regions for VI43 and VI39 and have therefore enabled the function of other sequences necessary for *TK* gene expression to be understood. Since genomic coding sequences for VI43 span approximately 1,110 bp, and assuming the location of protein synthesis initiation is correct, it is clear that introns of significant size cannot exist in this gene. Furthermore, the absence in adjacent sequences of other mRNA's with similar abundance or kinetics of synthesis indicates that the *TK* gene is controlled as an independent unit rather than one of a group of coordinately expressed products. Despite the high GC content and requirement for a viral polypeptide in infected cells (24, 35, 36), the *TK* gene exhibits all the postulated signals for RNA polymerase binding and initiation of transcription.

An important finding from mapping *Bam*HI *p*-specific mRNA's is the identification of two polypeptides whose mRNA's share coding sequences. Whether VI43 and VI39 are produced from the same translational reading frame cannot be determined until further analysis of these polypeptides has been performed. Nevertheless, mechanisms by which VI39 might be related to VI43 can be considered. The possibility of proteolytic cleavage of VI43 was eliminated by the finding that the relative proportions of the two polypeptides did not change during a chase of up to 20 h in vitro (data not shown). Premature termination of protein synthesis also seems unlikely, since *Sma*I fragment B partially inhibits production of both VI39 and VI43, even though it cannot overlap sufficient coding sequences to mask a pretermination site. The other possibilities appear to be splicing of coding sequences from VI43 mRNA or false initiation of protein synthesis at a site 100 to 150 bp downstream from the VI43 initiation codon.

The evidence currently available does not support the concept of splicing VI43 mRNA to generate VI39 mRNA. These mRNA's could not be separated by sucrose density gradient centrifugation or polyacrylamide gel electrophoresis under denaturing conditions (data not shown), and no band 100 to 150 bp smaller than VI43 mRNA could be detected when pTK1 DNA was

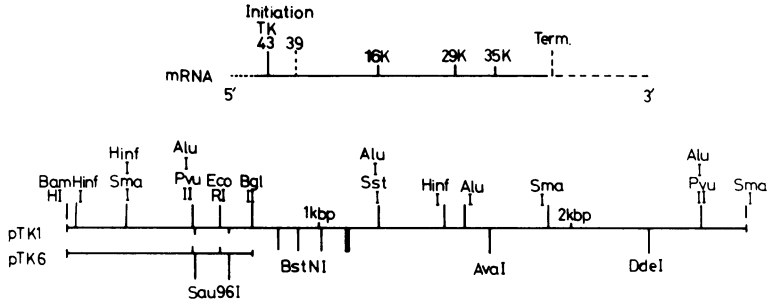

FIG. 10. *Summary of mRNA mapping data. Sequences which arrest translation under standard conditions are shown in solid lines; those which require altered conditions or partially arrest are shown in broken lines. Points 16,000, 29,000, and 35,000 molecular weight units from the proposed VI43 initiation codon are marked and should be compared to the AluI, HinfI and AvaI sites which generated putative truncated polypeptides of these sizes. The proposed termination site for VI43 and VI39 and initiation site for VI39 are also indicated. Restriction sites above the lines are from complete maps of pTK1; those below have been identified only in digests of pTK1 subfragments.*

used to probe fractionated RNA on Northern blots (J. B. Clements and N. M. Wilkie, manuscript in preparation). Such experiments might not succeed because the expected mRNA size reduction is less than the average length of the heterogeneous polyadenylic acid tract. Any splice would be more than 270 bp downstream from the *BglII* site, since removal of 100 to 150 bp in this region would prevent arrest of VI39 synthesis by one or more of the small *BglII-SstI-BstNI* fragments C, D, or E. Alternatively, a situation in which ribosomes occasionally read through the usual initiation codon is compatible with the data presented here, but rigorous discrimination between the possibilities will require detailed peptide mapping of VI43 and the minor VI39 band. It should be noted that ribosome attachment for both VI43 and VI39 occurs near the 5′ terminus (18), since pTK6 DNA can arrest synthesis of both polypeptides.

Figure 10 summarizes the mapping of TK, VI43, and VI39 mRNA's, and indicates the putative initiation codon for VI39.

At the recent Cold Spring Harbor Herpesvirus Workshop, where this work was presented, the complete sequence of the HSV-1 *TK* gene was reported by M. Wagner, J. Sharp, and W. C. Summers, and by S. McKnight. Analysis of strain CL101 shows that the *SmaI*-B overlaps coding sequences by 18 bp, and that the next ATG codon from the proposed VI43 initiator is 130 bp downstream and in phase (M. Wagner, personal communication).

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