Mapping and Identification of the Vaccinia Virus Thymidine Kinase Gene

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The thymidine kinase gene of vaccinia virus (VV) was mapped on the viral genome by using cloned fragments of the viral DNA to hybridize to early viral mRNA. Individual DNA fragments that represented about half of the viral genome were assayed, both for their ability to arrest the cell-free synthesis of active VV thymidine kinase and for their ability to select functional mRNA for the viral enzyme. Both activities were located in *Hind*III fragment J, which maps near the middle of VV DNA and contains about 2.6% of the genome (4,800 base pairs). This DNA fragment encodes four known early polypeptides, and to determine which of these was thymidine kinase, early VV mRNA was fractionated by sucrose gradient centrifugation and used to direct cell-free synthesis of the active enzyme. The thymidine kinase mRNA cosedimented with several species that encoded polypeptides in the molecular weight range 15,000 to 25,000. Hybridization of these mRNAs to *Hind*III-J DNA selected a message that directed the synthesis of thymidine kinase and a single polypeptide with an apparent molecular weight of 19,000. The native molecular weight of VV thymidine kinase is about 80,000, so these data indicate that, unlike thymidine kinase from several other sources, the active VV enzyme is probably a tetramer of 19,000-molecular-weight subunits.

The DNA genome of vaccinia virus (VV) contains about 185,000 base pairs and encodes approximately 200 polypeptides (7, 18). The sequence complexity of the viral mRNA made early in infection indicates that about half of the viral genes are expressed before replication of the viral DNA (2, 8), and analysis of the early viral polypeptides supports this conclusion (unpublished data). The early viral genes appear to be distributed over almost all regions of the genome, because early mRNA hybridizes to all but 1 of the 15 fragments produced by *Hind*III digestion of VV DNA (3). Translation of hybrid-selected mRNA populations confirms that the early and late genes are interspersed and has revealed the approximate map positions of about 75 early and 40 late polypeptides (1, 4). Among the early genes, two control classes have been distinguished. Transcription of genes in the immediate-early category is mediated by the virion RNA polymerase and does not depend on the expression of other viral genes. On the other hand, expression of the delayed-early genes requires one or more immediate-early viral functions. Late gene expression requires viral DNA synthesis (18). However, analysis of the polypeptides made throughout VV infection shows that there are many different kinetic patterns,

suggesting the existence of complex regulatory mechanisms (11, 21).

Among the early genes is one that codes for a viral thymidine kinase (tk; EC 2.7.1.7). This gene is expressed immediately upon infection, but is switched off about 4 h later, apparently by a translational repression mechanism that is mediated by the protein product of a viral gene (13-17). To examine this control mechanism further, we recently developed an assay for VV tk mRNA based on its ability to direct active enzyme synthesis in a reticulocyte lysate (12). Using this assay to measure the levels of tk mRNA throughout infection, we confirmed that shutoff occurred at the translational level and showed that it was accompanied by, but did not depend on, the functional inactivation of tk mRNA (11). It is clear that further analysis of this system would benefit from the development of physical assays for tk mRNA and for the enzyme itself, to complement the functional assays already available. A physical assay for tk mRNA would require the identification, mapping, and molecular cloning of the tk gene to provide a convenient hybridization probe. The results presented in this paper show that the structural gene for VV tk is contained within *Hind*III-J, a 4,800-base-pair fragment which con-

tains sequences located near the middle of the viral DNA (1, 15). A physical assay for the tk enzyme would require the characterization of its constituent polypeptide(s). Its native molecular weight is 80,000 to 85,000, and analysis of partially purified preparations has revealed the presence of two major viral polypeptides of molecular weight 40,000 to 42,000 (14). Since other tk enzymes are dimers of identical subunits with molecular weights of about 40,000 (10, 25), it has seemed likely that one or both of these VV polypeptides constituted the viral enzyme, although rigorous identification has not been achieved. The results reported in this paper indicate that, contrary to expectation, VV tk is composed of subunits with an apparent molecular weight of about 19,000.

MATERIALS AND METHODS

Cells and virus. The growth and maintenance of mouse L-cells that lacked thymidine kinase (Ltk⁻ cells) and their infection with VV strain WR have been described previously (11, 12).

Purification and translation of VV mRNA. mRNA was isolated from VV-infected Ltk⁻ cells as described before (11, 12). To enhance the concentration of immediate-early viral mRNAs, including tk mRNA (11), cells were infected in the presence of cycloheximide (100 µg/ml), and mRNA was extracted 5 h later. Polyadenylated mRNA was fractionated by sedimentation through 12.5-ml 5 to 20% sucrose gradients centrifuged at 38,000 rpm for 8 h at 25°C. The mRNA in each 0.4-ml fraction was collected by ethanol precipitation in the presence of 10 µg of calf liver tRNA and analyzed for its ability to direct cell-free protein and tk synthesis (12). The mRNA fractions that encoded tk were pooled and reprecipitated with ethanol. Typically, 2.5×10^8 infected cells yielded about 1.4 mg of total RNA, of which about 12% bound to oligodeoxythymidylic acid-cellulose. About 90% of the functional VV tk mRNA was polyadenylated.

Cell-free protein synthesis was carried out in mRNA-dependent reticulocyte lysates as described before (12), and after RNase digestion, the products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (18). Nonradioactive translation products were assayed for tk activity as described before (12).

Preparation of plasmid DNA. Recombinant plasmids containing VV *Hind*III restriction fragments D through O (which together comprise about 50% of the VV genome) in the *Hind*III site of pBR322 were constructed and generously provided by B. Moss (National Institutes of Health) (1). Plasmids were purified by the methods of Hardies et al. (9) from amplified cultures of *Escherichia coli* HB101 grown in the presence of 20 µg of ampicillin per ml. The identity and purity of each plasmid preparation were checked by digestion with *Hind*III endonuclease (Bethesda Research Laboratories, Gaithersburg, Md.) and analysis of the products by electrophoresis on 0.8% agarose gels (1).

Hybrid arrest and hybrid selection of VV tk mRNA. Hybrid-arrested translation was performed by the procedure of Paterson et al. (19). For hybrid selection of

VV tk mRNA, 10 µg of the purified DNA fragments were bound to nitrocellulose filters and used to select viral mRNAs as described by Ricciardi et al. (22).

RESULTS

Hybrid arrest of tk synthesis. mRNA extracted from VV-infected Ltk⁻ cells early in infection directs the cell-free synthesis of active VV tk in mRNA-dependent rabbit reticulocyte lysates (12). This provides a specific assay for VV tk mRNA that can be used to map the structural gene for the enzyme. Twelve of the fifteen DNA fragments produced by *Hind*III digestion of VV DNA (Fig. 1) have been inserted into plasmid pBR322 and cloned in *E. coli*, and these clones were generously made available to us (1). Plasmid DNAs from each of these 12 clones were tested for their ability to arrest the cell-free synthesis of active tk after hybridization to early VV mRNA (19). The results showed that only the recombinant plasmid that contained *Hind*III fragment J of VV DNA profoundly inhibited enzyme synthesis (Table 1). In some experiments, DNA from the other recombinant plasmids gave partial nonspecific inhibitions, but the magnitude of these effects did not compare with the inhibition produced by fragment J. In particular, when plasmid DNA that was more highly purified than that used in Table 1 was employed, neither of the DNA fragments adjacent to J in the *Hind*III map inhibited tk synthesis (Fig. 2). The degree of inhibition of tk synthesis by *Hind*III-J DNA depended on the amount of plasmid DNA that was used in the hybridization reaction (data not shown), but no inhibition of total protein synthesis was observed (Fig. 2). Denaturation of the J DNA-mRNA hybrid before cell-free translation substantially restored the tk mRNA activity (Fig. 2).

Hybrid selection of tk mRNA. On the basis of the results of hybrid-arrested enzyme synthesis, DNA fragment J and its two neighboring fragments L and H in the *Hind*III map of VV DNA were tested for their ability to select tk mRNA by hybridization to a mixture of early viral mRNAs. The three viral DNA fragments were purified by agarose gel electrophoresis of *Hind*III digests of the appropriate recombinant plasmids, immobilized individually by binding to nitrocellulose filters, and used to hybridize to total early VV mRNA (22). After thorough washing of the filters, the retained mRNAs were eluted with 90% formamide at 65°C and compared with the unbound mRNAs for their abilities to direct protein synthesis and tk synthesis in vitro. Among the bound mRNAs, only the preparation that had been selected by hybridization to fragment J demonstrated tk mRNA activity (Table 2). Moreover, fragment J was unique in its ability to deplete the unbound mRNA

FIG. 1. *Hind*III restriction site map of VV strain WR genome (1).

fraction of tk mRNA activity. DNA fragments G and K were also tested and found to be unable to select tk mRNA (data not shown). Thus, the results of hybrid arrest and hybrid selection of tk mRNA activity indicated that of the 12 *Hind*III fragments D through O, only fragment J could hybridize to tk mRNA.

Sucrose gradient analysis of tk mRNA. Translational mapping studies using early VV mRNA selected by hybridization to cloned *Hind*III DNA fragments have shown that fragment J encodes all or part of four early polypeptides (1). Two major species with apparent molecular weights of 41,000 and 17,000 and a relatively minor 21,000-molecular-weight species appear to be encoded entirely within fragment J, whereas a 110,000-molecular-weight delayed-early polypeptide, which may be the viral DNA polymerase, is encoded in sequences which span the J-H boundary. To determine which of these polypeptides was tk, early VV mRNA was subjected to sucrose gradient centrifugation, and individual fractions were used to direct cell-free

protein and enzyme synthesis (Fig. 3A). The polypeptide products of cell-free translation were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3B). Most, but not all, of the major polypeptides synthesized in this experiment were viral in origin, because early viral mRNAs were translated more efficiently and encoded a smaller spectrum of polypeptides than bulk cellular mRNAs. Furthermore, most, but not all, of the polypeptides were encoded in mRNAs with narrow size ranges that we appropriate for the molecular weight of the encoded polypeptide. This is indicated by the dominant diagonal distribution of the majority of polypeptides in Fig. 3B. Nevertheless, a few polypeptides were encoded in mRNAs that sedimented anomalously fast or, in rare cases,

TABLE 1. Hybrid arrest of cell-free synthesis of VV tk^a

DNA fragment	Relative tk activity (%)
None	100
<i>Hind</i> III-D	67.3
<i>Hind</i> III-E	88.2
<i>Hind</i> III-F	56.9
<i>Hind</i> III-G	57.7
<i>Hind</i> III-H	52.0
<i>Hind</i> III-I	60.1
<i>Hind</i> III-J	6.8
<i>Hind</i> III-K	81.1
<i>Hind</i> III-L	53.3
<i>Hind</i> III-M	57.0
<i>Hind</i> III-N	59.5
<i>Hind</i> III-O	59.7

^a mRNA was extracted from VV-infected cells at 5 h after infection in the presence of cycloheximide and hybridized to *Hind*III digests of recombinant plasmids carrying the indicated VV DNA fragments (19). After overnight hybridization, the RNA-DNA mixtures were used to direct cell-free protein synthesis, and the translation products were assayed for tk activity. The results shown are the averages of two experiments, in which the 100% value (no added DNA) corresponded to an average 61-fold enhancement above the endogenous tk activity of the reticulocyte lysates.

FIG. 2. Hybrid arrest of the cell-free synthesis of: (a) VV tk, and (b) VV immediate-early proteins by *Hind*III fragments L, J, and H. The hybridization of immediate-early viral mRNA to the cloned *Hind*III fragments L, J, and H was performed as described in the text and in the legend to Table 1. Half of each hybridization mixture was translated without denaturation (stippled bars), and half was translated after denaturation at 100°C for 3 min to release the hybridized mRNA (open bars). Cell-free protein synthesis was measured by [³⁵S]methionine incorporation, and cell-free tk synthesis was measured by the phosphorylation of [³H]thymidine (12).

TABLE 2. Hybrid selection of VV tk mRNA^a

Immobilized DNA fragment	Assay	Incorporated cpm directed by:	
		Bound mRNA	Unbound mRNA
<i>Hind</i> III-L	Protein synthesis	12,100	220,900
	tk synthesis	1,920	108,700
<i>Hind</i> III-J	Protein synthesis	19,780	186,700
	tk synthesis	97,500	1,530
<i>Hind</i> III-H	Protein synthesis	11,950	166,150
	tk synthesis	2,200	104,850

^a Immediate-early VV mRNA was fractionated by sucrose gradient centrifugation as shown in Fig. 3, and the fractions that directed tk synthesis were pooled. From this fraction, mRNAs were selected by hybridization to immobilized *Hind*III fragments L, J, or H (22). The bound mRNAs were eluted from the washed filters, and they and the unbound mRNAs were used to direct cell-free protein synthesis. The translation products were assayed by incorporation of [³⁵S]methionine (³⁵S counts per minute per 2 μ l; background = 410 cpm) and by measurement of their tk activities (³H counts per minute per 8 μ l; background = 1,200 cpm).

anomalously slowly. These polypeptides lie below and above the dominant diagonal, respectively (Fig. 3B).

mRNA for tk cosedimented with mRNAs encoding several polypeptides with molecular weights of 15,000 to 25,000. No polypeptides with molecular weights greater than 35,000 were detectable, even among the products directed by the leading edge of the peak of tk mRNA activity. The apparent molecular weight of the tk mRNA was calculated on the basis of a comparison of its rate of sedimentation relative to those of the mRNAs that encode the N, NS, M, and G proteins of vesicular stomatitis virus. For this experiment, the VV and vesicular stomatitis virus mRNAs were mixed and denatured in 85% formamide before being subjected to sedimentation through a 5 to 25% sucrose gradient containing 70% formamide. Individual fractions were tested for their ability to direct cell-free protein and enzyme synthesis. tk mRNA sedimented with an apparent molecular weight of 0.23×10^6 , which corresponds to a length of about 700 nucleotides (data not shown). Its sedimentation rate was significantly slower than that of the vesicular stomatitis virus M and NS mRNAs, which contain 956 and 940 nucleotides (including an estimated 125 nucleotides contributed by polyadenylate tails) and encode proteins with molecular weights of 26,064 and 25,110, respectively (6, 23).

Polypeptide composition of VV tk. The measurements of the apparent molecular weight of tk mRNA suggested that the enzyme was proba-

bly composed of one of the two smaller early polypeptides encoded by *Hind*III fragment J. To examine this question further, the peak of tk mRNA from a sucrose gradient like that shown in Fig. 3 was hybridized to immobilized fragment L, J, or H DNA, and the polypeptides directed by the bound and unbound mRNA fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). mRNA selected by hybridization to DNA fragment J directed the synthesis of a single major polypeptide with an apparent molecular weight of 19,000. On the other hand, DNA fragments L and H retained mRNAs that directed the synthesis of either no detectable polypeptides (fragment L) or of a single major polypeptide with an apparent molecular weight of 35,000 (fragment H). These results confirmed previous observations that *Hind*III-L contains no immediate-early genes (1, 3) and that fragment H encodes an immediate-early polypeptide with a molecular weight of about 40,000 (1).

DISCUSSION

The enzymes of the nucleoside salvage pathways provide versatile systems for genetic manipulation because they are generally encoded in nonessential genes that can be subjected to either positive or negative selection pressures. The presence of genes for one of these enzymes, tk, within the genomes of some herpesviruses and poxviruses adds to the advantages of the system, as illustrated by the exploitation of the herpes simplex virus tk gene for DNA-mediated gene transfer into tk⁻ cells (20). The VV tk gene has received less attention in this respect, partly because VV DNA appears to be unable to transform tk⁻ cells biochemically to the tk⁺ phenotype (12). It remains to be seen whether the isolated VV tk gene is similarly incompetent. On the other hand, considerable attention has been focused on the control of VV tk expression during infection (11, 13, 16, 17). The abrupt shutoff that occurs about 4 h postinfection is one of the clearest known examples of the control of viral gene expression at the translational level, and its mechanism is therefore of considerable interest.

For these reasons we have undertaken a detailed study of the VV tk gene: its location, structure, expression, and control. The results presented above indicate that of the 12 *Hind*III fragments D through O of VV DNA, only fragment J contains nucleotide sequences that are complementary to VV tk mRNA. Between them, fragments D through O represent only about 50% of the VV genome, so it remained possible that a second tk gene might be located in *Hind*III fragment A, B, or C. Since we have not studied these fragments, we cannot elimi-

FIG. 3. (A) Fractionation of immediate-early VV mRNA by sucrose gradient centrifugation. mRNA was isolated from VV-infected cells 5 h after infection in the presence of cycloheximide, as described in the text. It was fractionated by sucrose gradient centrifugation, and the individual fractions were used to direct cell-free protein and tk synthesis. The sedimentation positions of tRNA and 18S and 28S rRNA are indicated. (B) The cell-free translation products, labeled by incorporation of [^{35}S]methionine, were resolved by electrophoresis on a 12.5% SDS-polyacrylamide gel (24). An autoradiograph of the dried gel is shown, and the migration positions and the molecular weights ($\times 10^{-3}$) of four marker proteins are indicated. The numbers above each gel lane correspond to the sucrose gradient fraction numbers.

nate the possibility that one or more of them contains sequences capable of hybridizing to tk mRNA. However, two lines of evidence indicate that VV contains only a single active tk gene: the ease with which tk⁻ mutants can be isolated (5) and the observation that all such mutants so far

tested can be rescued by the same fragment of VV DNA, *Hind*III fragment J (26). Thus, the physical evidence presented above complements the genetic evidence derived from marker rescue experiments to establish that the structural gene for VV tk is located in *Hind*III frag-

FIG. 4. SDS-polyacrylamide gel electrophoresis of the translation products of gradient-purified and hybrid-selected VV mRNA. Immediate-early VV mRNA was fractionated by sucrose gradient centrifugation as shown in Fig. 3A, and the peak of tk mRNA activity was hybridized to purified immobilized VV *Hind*III-L, -J, or -H DNA (22). The mRNA which hybridized and that which failed to hybridize were translated separately. The translation products were analyzed by electrophoresis on a 12% SDS-polyacrylamide gel (24), and an autoradiograph of the dried gel is shown. Translation products were as follows: lane 1, no added mRNA; lane 2, total gradient-purified mRNA; lane 3, mRNA that hybridized to L DNA; lane 4, mRNA that failed to hybridize to L DNA; lane 5, mRNA that hybridized to J DNA; lane 6, mRNA that failed to hybridize to J DNA; lane 7, mRNA that hybridized to H DNA; lane 8, mRNA that failed to hybridize to H DNA. The molecular weights ($\times 10^{-3}$) of the translation products of brome mosaic virus and vesicular stomatitis virus mRNA are indicated as markers.

ment J at 42.5 to 45.1 map units in the *Hind*III map of VV DNA (Fig. 1).

The molecular weight of fragment J is about 3.2×10^6 , which corresponds to about 4,800 base pairs. Translational mapping studies have assigned three immediate-early genes to this fragment: a major species with an apparent molecular weight of about 17,000 and less abundant species with molecular weights of 21,000 and 41,000 (1). In addition, a 110,000-molecular-weight delayed-early polypeptide, which is a good candidate for the viral DNA polymerase, was encoded in a gene that spans the J-H

boundary. The other boundary, between *Hind*III fragments L and J, is apparently spanned by a gene for one of the several late polypeptides that have been assigned to fragment J (1). Since VV tk is an immediate-early gene, the best candidates for the polypeptide that constitutes the enzyme are the 41,000-, 21,000-, and 17,000-molecular-weight species. The measurements of the molecular weight of tk mRNA (Fig. 3) indicate that it contains about 700 nucleotides, including its polyadenylate tail. This size is appropriate to encode a polypeptide in the molecular weight range of 15,000 to 25,000 but could not accommodate a 41,000-molecular-weight species. Of course, the RNA and protein molecular weights were derived by nonrigorous methods and may be inaccurate. Nevertheless, it is inescapable that the peak of tk mRNA activity, after sucrose gradient centrifugation, directed the synthesis of polypeptides which migrated on SDS-polyacrylamide gels in the molecular weight range of 15,000 to 25,000 and which lacked detectable amounts of the 41,000-molecular-weight species (Fig. 3). These results appear to eliminate the 41,000 molecular-weight polypeptide as a candidate for tk. On the other hand, the 21,000- and 17,000-molecular-weight species are the right size to be encoded in 700-nucleotide mRNAs. Moreover, the only major polypeptide that was synthesized in response to gradient-purified, J-selected mRNA had a molecular weight of 19,000 in our hands (Fig. 4). We conclude that this polypeptide is probably the only viral component of VV tk, although its relationship to the 21,000- or 17,000-molecular-weight polypeptides of Belle Isle et al. (1) is unclear. Since the native molecular weight of the enzyme is 80,000 to 85,000, active tk must either contain several copies of the 19,000-molecular-weight species or, perhaps, cellular components in addition to the viral polypeptide. We consider the latter possibility less likely, since the native molecular weight of VV tk made in a reticulocyte lysate is the same as that from infected mouse L-cells (12).

This polypeptide assignment disagrees with that of Kit et al. (14), who analyzed the labeled polypeptide composition of VV tk that was partially purified from the cytoplasm of [35 S]methionine-labeled VV-infected cells. These authors concluded that the enzyme was probably a dimer of 40,000- to 42,000-molecular-weight subunits, like the herpes simplex virus and the mouse L-cell enzymes (10, 25). Possible explanations for the discrepancy are that the 40,000- to 42,000-molecular-weight polypeptide was a contaminant or, conceivably, that the virus strain (IHD) that Kit et al. were studying encodes a tk enzyme which differs in molecular weight from that encoded by strain WR.

Neither of the *Hind*III fragments that adjoin J was able to hybridize to tk mRNA. Moreover, other genes that span both junctions have been identified (1). These observations indicate that the tk gene lies completely within *Hind*III fragment J, and this conclusion is supported by recent analyses of subfragments of J, which show that the gene lies between about 0.5 and 1.2 kilobases from the L-J boundary (manuscript in preparation). Precise mapping and characterization of the VV tk gene will enable us to construct appropriate tk-specific hybridization probes for use in the further analysis of the mechanisms that control tk expression during infection and to determine what nucleotide sequences are required to render the VV tk gene competent to transform tk⁻ cells to the tk⁺ phenotype.

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