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Characterization of the mRNA for herpes simplex virus thymidine kinase by cell-free synthesis of active enzyme

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ABSTRACT

The cytoplasmic mRNA which codes for the herpes simplex virus specific thymidine kinase (TK) is polyadenylated and is 14.5s in size. This corresponds to an RNA of 1400 nucleotides. The TK polypeptide is about 42,000 daltons, which requires 1100 nucleotides. We conclude that the cytoplasmic mRNA is monocistronic. The reticulocyte lysate cell-free translation system synthesizes enzymatically active HSV-TK which can be assayed with high specificity and sensitivity by use of ^{125}I-iododeoxycytidine as a substrate.

INTRODUCTION

The synthesis, processing, translation and degradation of messenger RNA in mammalian cells are highly complex processes (1,2). The use of *in vitro* protein synthesis systems allows one to study the metabolism of a variety of messenger RNAs. Studies of specific mRNAs have been limited to viral mRNAs such as those from oncornavirus, adenovirus and papovirus (3,4,5), and those mRNAs which code for abundant proteins, such as hemoglobin and ovalbumin (6,7). To date, interferon mRNA (8) and herpes simplex virus thymidine kinase (HSV-TK) mRNA (9) are the only ones which have been translated in cell-free systems to yield biologically or enzymatically active protein.

In a previous report we showed that the TK polypeptide was synthesized *in vitro* in a preinitiated cell free system (10). The presence of polyribosomes from infected cells which were significantly contaminated with endogenous viral TK prevented detection of newly synthesized enzymatically active TK. In the present study we have employed the micrococcal nuclease treated reticulocyte system of Pelham and Jackson (11) to synthesize enzymatically active TK using total cytoplasmic RNA from HSV-infected cells. Unlike cellular TK, HSV-TK can be assayed with high specificity and sensitivity using ^{125}IdC as a substrate (12). We have used the

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system to determine the size of the cytoplasmic mRNA for HSV-TK and to show that the messenger RNA is polyadenylated.

MATERIALS AND METHODS

Growth of Cells and Virus: Herpes simplex virus, strain Cl 101 and thymidine kinase negative mutants (TK^-) were propagated on Vero cells at an m.o.i. of 0.05 to 0.1 pfu/cell. Infectious virus was released from cell pellets by gentle sonication and was stored at -70°C.

Preparation of Cytoplasmic RNA: Mouse $LMTK^-$ cells were grown in roller bottles and infected with wild type TK^+ virus at an m.o.i. of 15-20 pfu/cell. After 7 to 8 hours, infected cells were harvested onto crushed, frozen PBS (phosphate buffered saline) and centrifuged for 5 min at 700 xg. Cells were washed once with ice-cold PBS, after which they were resuspended in 4 volumes of tris-saline (0.01 M Tris HCl, pH 7.4;0.15 M NaCl; 0.002 M $MgCl_2$) containing 0.5% NP40 (13). After 5 min on ice, the cells were lysed in a Dounce homogenizer. Nuclei were removed by centrifugation for 5 min at 1500 xg. The supernatent fraction was added to an equal volume of TSE buffer (0.01 M Tris-HCl, pH 7.6; 0.15 M NaCl, 0.005 M EDTA) which contained 1 percent SDS, and was extracted three times with an equal volume of phenol: chloroform: iso-amyl alcohol (24:24:1) saturated with TSE buffer, and then extracted twice with chloroform. The nucleic acid solution was made 0.15 M sodium acetate and the nucleic acids were precipitated with 2 volumes of ethanol. After overnight storage at -20°C, the RNA precipitate was collected by centrifugation, briefly dried *in vacuo*, resuspended in water at a concentration of 10-20 mg/ml and stored at -20°C.

Cell-Free Translation: The micrococcal nuclease-treated reticulocyte lysate system was prepared as described by Pelham and Jackson (11). The protein synthesis system was made up as follows: 0.40 ml reticulocyte lysate, 4 µl of 5 mg/ml creatine phosphate kinase; 8 µl of mM hemin (Eastman), 24 µl of 2M KCl with 10 mM $MgCl_2$, 24 µl of a solution with 5mM of 19 amino acids without methionine and 24 µl of 0.2 M creatine phosphate. The lysate mixture was made 1 mM in $CaCl_2$ and 20 µg/ml in micrococcal nuclease (Worthington) and incubated for 13 minutes at 20°C. The nuclease digestion was terminated by the addition of EGTA to give a final concentration of 2 mM. Rabbit liver tRNA (Gibco) was added to give 15 µg/ml. Aliquots of 13 µl were added to tubes containing 1-3 µl of total cytoplasmic RNA from herpes simplex virus infected cells and 14 µCi of [^{35}S] methionine (Amersham, 600-1200 Ci/m mole). Reaction mixtures were incubated at 30°C for 80 minutes unless otherwise indicated, after which

3 µl was treated with KOH, precipitated with trichloracetic acid, filtered and counted as described (10). The remaining part of the reaction was treated for 10 minutes at 30°C with 3 µl of a solution containing 0.2M EDTA and 200 µg/ml pancreatic RNase. Four volumes of SDS-sample buffer for polyacrylamide gel electrophoresis was added and this mixture was heated at 95°C for 3 min; 4 to 5 µl was subjected to electrophoresis on polyacrylamide gels as previously described (10).

Purification of ^{125}IdC: An aqueous solution of [^{125}I]dC (iododeoxycytidine) (12) was dried and taken up in 5-10 µl H_2O and applied to DEAE-paper. It was chromatographed in 0.2 M NH_4HCO_3 and 0.005 M $Na_2B_4O_7$. The chromotograph was autoradiographed briefly, the [^{125}I]dC spot (RF=0.68) was cut out and eluted with water. This material was dried again and redissolved in water at $1x10^8$ cpm/ml. This purification step was necessary to remove a radioactive breakdown product (RF=0.27) which accumulated and resulted in very high backgrounds.

In Vitro Phosphorylation of [^{125}I]dC: In vitro protein synthesis was carried out as described in the previous section with the exception that [^{35}S]-methionine was replaced by 0.25 mM unlabeled methionine; 1.25 mM ATP and 10% glycerol were also present. In some experiments, additional $MgCl_2$ was added. 40 µl protein synthesis reactions were incubated for 80 min at 30°C, after which they were diluted 20% by the addition of the following components to give a final concentration of 0.1 M sodium phosphate, pH 6.0, 0.01 M ATP, 0.01M Mg Ac_2, 0.025 M NaF, and $1.2x10^7$ cpm/ml [^{125}I]dC. After 0,3 and 6 hours of incubation, 10 µl of the assay mixture was diluted with 40 µl H_2O, boiled for 2 minutes and centrifuged for 2 minutes at 10,000 xg. The supernatant (45 µl) was applied to DEAE-paper (2x2 cm). The papers were washed individually four times in 20 ml of 0.04 M NH_4HCO_3 and 2 mM KI at 37°C with slow shaking. They were then washed once with water and twice with 95% ethanol. Papers were dried and counted in a liquid scintillation counter. Unwashed, dried papers with samples from each assay were used to determine the total isotope in each reaction.

Definition of TK Activity: The isotopic assay for HSV-TK which we have employed makes use of [^{125}I]dC which we have synthesized. The small scale of our syntheses prevented us from being able to determine accurately the specific activity of the [^{125}I]dC. We could calculate the expected specific activity if we assume that the iodine-125 was carrier-free, but the separation of [^{125}I]dC from the excess dC was difficult and a few percent of the dC may contaminate the IdC. Since both compounds are

substrates, the "effective specific activity" varied between different preparations depending on the level of dC contamination. For this work we adopted a convention which was internally consistent within any one set of experiments. We determined the concentration of [^{125}I]dC which gave substrate-excess kinetics. We then used a constant, but not precisely known, concentration of IdC in any given experiment. The incorporation of radio-iodine was calculated directly as counts per minute determined under a standard set of conditions for radioactivity measurement. The rate of incorporation was determined over a three to six hour period. For the purpose of the in vitro synthesis of TK, we report TK activity in terms of cpm incorporated per hour in 10 µl under the standard assay conditions.

Sedimentation of RNA: RNA was fractionated on sucrose gradients containing formamide as described by Anderson et al (13). RNA was denatured by incubation of 100 µl of RNA (800 µg) with 100 µl of 10X buffer (1 M LiCl, 0.05 M EDTA, 2% sodium dodecyl sulfate, 0.01 M Tris HCl, pH 7.4) and 0.90 ml of deionized formamide at 37°C for 5 minutes. The RNA was diluted with an equal volume of 1X buffer (10X buffer diluted with 9 volumes of water) and the resulting 2.0 ml of RNA solution (16 A_{260} units of RNA) was loaded onto a 35 ml, 5% to 20% linear sucrose gradient made up in 1X buffer containing 50% formamide (v/v). The gradients were centrifuged in the Spinco SW27 rotor at 25,000 rpm for 43 hours at 4°C. The gradients were collected into 1.6 ml fractions and the optical absorbance at 260 nm was determined. The fractions were precipitated with 2 volumes of ethanol after addition of sodium acetate to a concentration of 0.5M and E. coli 16S rRNA to 15 µg/ml. After a second ethanol precipitation, the remaining fluid was removed by brief drying in vacuo. The RNA was dissolved in 15 µl water and 3 µl of each fraction was used for in vitro protein synthesis with [^{35}S]-methionine and the products were analyzed by gel electrophoresis. Six µl of RNA from various fractions was used for in vitro synthesis of active viral thymidine kinase.

Immunological Procedures: Antiserum against HSV-infected rabbit kidney cells was kindly provided by Dr. Alec Buchan and antiserum against highly purified HSV-TK (14) was a gift from Dr. Y-C. Cheng. To test for inactivation of TK activity by antisera, 0-5 µl of sera were added to the reaction mixture after protein synthesis. Phosphate buffered saline was added so that each sample received 5 µl total increase in volume. The samples with PBS or the various amounts of antisera were incubated at 30°C for 30 minutes and then assayed for TK activity as described above.

RESULTS

Enzymatically Active HSV-TK is Synthesized In Vitro: Since the cell-specific TK will not phosphorylate [^{125}I]dC (12), the use of [^{125}I]dC as a substrate allowed us to assay only the HSV-specific TK. Table 1 shows that the conditions which give maximal protein synthesis as determined by [^{35}S]-methionine incorporation are not identical to those that give maximal TK enzyme synthesis. Different reticulocyte lysates and mRNA preparations varied in their ability to synthesize TK. The optimal conditions for TK synthesis in different lysates were determined by varying the magnesium concentration. Since the ATP concentration was increased in order to help stabilize the TK activity, the system as we use it seems to require more magnesium than originally described by Pelham and Jackson(11).

Increasing amounts of total cytoplasmic RNA from HSV infected cells were added to the in vitro system and the results are shown in Figure 1. The amount of active TK synthesized was directly proportional to the amount of RNA added. The time course for TK synthesis was followed by assay of TK activity. Figure 2 shows that TK is synthesized continuously during a 70 minute incubation. The total protein synthesis ([^{35}S]-methionine incorporation) was approximately parallel to the TK synthesis.

Once the protein synthesis was terminated by cycloheximide, additional ATP-Mg, NaF, phosphate buffer and [^{125}I]dC were added and the incubation continued. The phosphorylation of [^{125}I]dC was measured in aliquots withdrawn at various times. Figure 3 shows that the formation of IdCMP continues in a linear fashion for 3 to 6 hours. The linearity of this assay

Mg^{++}	TEMPERATURE			
	25°C		30°C	
	Activity	^{35}S-met	Activity	^{35}S-met
0.5 mM	350	4700	270	8000
1.5 mM	580	3000	1070	4000

Table 1: Effect of temperature and magnesium concentration on the synthesis of enzymatically active TK and [^{35}S]-methionine labeled protein in reticulocyte lysates programmed with cytoplasmic RNA from HSV-infected cells. Activity is expressed in cpm/hr/10µl and [^{35}S]-methionine incorporation is cpm/µl.

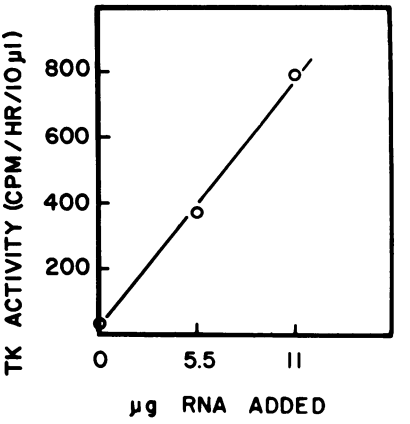

Figure 1: Proportionality of active TK synthesized in vitro with increasing amounts of cytoplasmic RNA from HSV-infected cells. 40 µl protein synthesis reactions were programmed with different amounts of total cytoplasmic RNA from HSV-infected cells. The amount of active TK enzyme was assayed as described in Materials and Methods. Reactions were carried out at 30 °C and with 0.5 mM Mg.

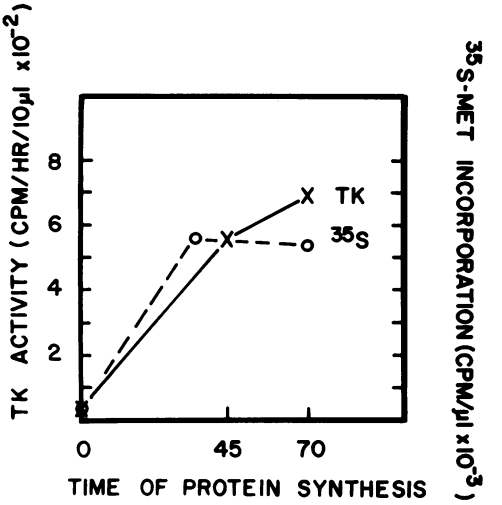

Figure 2: Time course of in vitro synthesis of active TK enzyme and [^{35}S]-methionine labeled proteins in reticulocyte lysates programmed with cytoplasmic RNA from HSV-infected cells. Cycloheximide was added at 0, 45 and 70 minutes to give a final concentration of 300 µg/ml. The amount of active TK made was then assayed or the [^{35}S]-methionine incorporation was determined. Reactions were carried out at 30 °C and with 0.5 mM Mg.

is similar to that observed for HSV-TK synthesized in vivo (15). We found that the addition of phosphate buffer (9) was essential; substitution of Tris-HCl buffer resulted in no measurable activities. We speculate

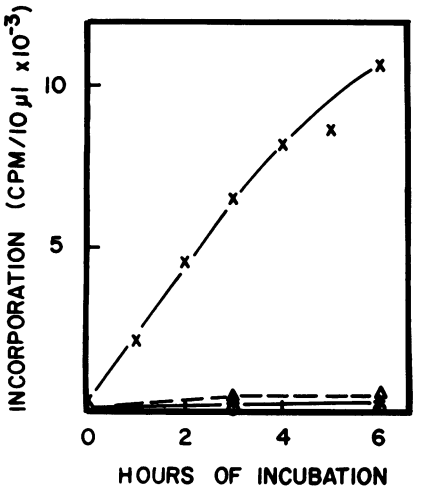

Figure 3: Time course of phorphylorylation of [^{125}I]dC by HSV-TK made in vitro (X). 10 µl samples were withdrawn from the assay mixture at the times indicated, and the radioactivity which bound to DEAE was determined. Protein synthesis reactions programmed without added HSV RNA (+) and incubations in the absence of reticulocyte lysate (0) and with RNA from uninfected LMTK$^-$ cells (Δ) are also shown. In this particular experiment 10,000 cpm represents about 40 percent of the total substrate. Such high incorporation may deplete the substrate and account for the non-linear incorporation after 4 hours. Reactions were carried out at 30 °C and with 1.5 mM Mg.

that the phosphate inhibits a phosphatase which destroys the product [^{125}I]-dCMP. The addition of NaF as an additional phosphatase inhibitor resulted in 20 to 50 percent higher incorporations and was essential for the extended incorporation over long incubation times.

Immunological Characterization of the In Vitro TK Activity: In order to confirm the identity of the enzyme which promoted the incorporation of IdC into IdCMP, we determined the ability of various antisera to inhibit the enzymatic activity synthesize in vitro. Antiserum raised against HSV-infected rabbit kidney cells, as well as antiserum made against highly purified HSV-TK both strongly inhibited the TK activity made in vitro (Figure 4). Control, pre-immune serum only partially blocked incorporation at high serum concentration.

Fractionation of Cytoplasmic RNA on Oligo dT-Cellulose: Samples of total cytoplasmic RNA from HSV-infected cells were subjected to chromatography on oligo dT-cellulose. The RNA was fractionated into bound and unbound RNA. The bound fraction was designated as "polyadenylated RNA" or poly A$^+$ RNA and the non-bound fraction was "nonpolyadenylated RNA" or

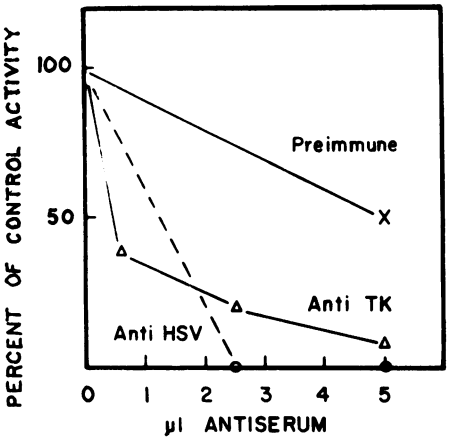

Figure 4: Immunologic inactivation of HSV TK enzymatic activity made in vitro in response to cytoplasmic RNA from HSV-infected cells. Various amounts of antiserum were added to the protein synthesis reaction after 80 minutes. After an additional 30 minutes of incubation at 30°C, the samples were assayed for TK activity. Results are normalized to the samples incubated without added antiserum (PBS added). Anti-TK serum was raised against purified HSV TK and anti-HSV serum was raised against sonicated HSV-infected cells.

poly A^- RNA. The total RNA in each fraction was resuspended in equal volumes and then equal samples were used to program protein synthesis in vitro. Only with the poly A^+ RNA could we synthesize active TK enzyme (Table 2). Polyacrylamide gel electrophoretic analysis confirmed that most of the TK mRNA activity was in the poly A^+ fraction.

Sedimentation Analysis of the HSV-TK mRNA: Total cytoplasmic RNA from HSV-infected cells was sedimented in sucrose gradients in the presence of 50 percent formamide to determine the sedimentation coefficient of the mRNA coding for HSV-TK (Figure 5). A sedimentation coefficient of 14.5 S was determined for the TK mRNA by reference to Vero cell 28 S, 18 S and 4 S RNAs and D. discoideum 25 S and 17 S rRNAs. If we assume that the TK mRNA has the same conformation in 50 percent formamide as do the markers, we can estimate the TK mRNA to be about 1400 nucleotides in length.

The RNAs from each fraction of the sucrose gradient programmed the synthesis of [^{35}S]-labeled proteins which were then analyzed by polyacrylamide gel electrophoresis in the presence of SDS (Figure 5). The polypeptide which we have previously identified as the HSV-TK by genetic and immunologic methods (10,15,16) is made in response to RNAs from the same region of the gradient which contains mRNA for active TK enzyme synthesis.

mRNA	TK Activity (cpm/hr/10 µl)
$LMTK^-$ (cell)	48
HSV-TK^+ (poly A^-)	56
HSV-TK^+ (poly A^+)	424

Table 2: In vitro translations were programmed with 20 µg of $LMTK^-$ cytoplasmic RNA, 52 µg HSV-TK^+ (poly A^-) RNA or 2 µg of HSV-TK^+ (poly A^+) RNA. Poly A^+ and poly A^- RNAs were prepared by chromatography on oligo-dT cellulose (Collaborative Research, Inc.). The sample was applied in 0.5 M NaCl, 0.01 M Tris HCl pH 7.4, and the column was washed with this solution. The poly A^- fraction was not retained; the poly A^+ fraction was eluted with 0.01 M Tris HCl, pH 7.4. RNAs in both fractions were precipitated with ethanol and resuspended in equal volumes of water. Equal volumes of these two RNA fractions were used in the translation assays. Reactions were carried out at 30°C and with 0.5 mM Mg.

DISCUSSION

The system for synthesis of enzymatically active HSV-TK described here is simple, sensitive and specific. A previous report by Preston (9) described synthesis of active HSV-TK in a system which required inhibition of endogenous cellular TK by dTTP in order to observe the newly made HSV-specific TK which is relatively resistant to dTTP. The method described here utilizes a substrate, IdC, which is highly specific for the viral enzyme. The potential for making very high specific activity [^{125}I]dC and the elimination of cellular activities without the use of inhibitors, which may be only partly effective or may partially inhibit viral TK, all suggest that the system we have described will be of particular value when assaying very low levels of TK mRNA or mutant TK mRNAs. In work to be published elsewhere (17), we have used this reticulocyte lysate system to show that some HSV-TK deficient mutants can be suppressed in vitro by yeast suppressor tRNAs.

The product made in vitro is similar to the enzyme made in vivo in infected cells in that it uses IdC efficiently as a substrate and is inactivated by anti HSV-TK antisera. Control experiments with mRNA from TK-negative HSV mutant-infected cells showed no synthesis of TK activity even though there were [^{35}S]-labeled HSV proteins made (except for the TK polypeptide) as determined by gel electrophoretic analysis (data not shown).

The size of the active TK enzyme is about 70,000 daltons as determined by gel filtration (16). The TK polypeptide has been determined to be about 42,000 daltons (15,16). Thus, it is most likely that the

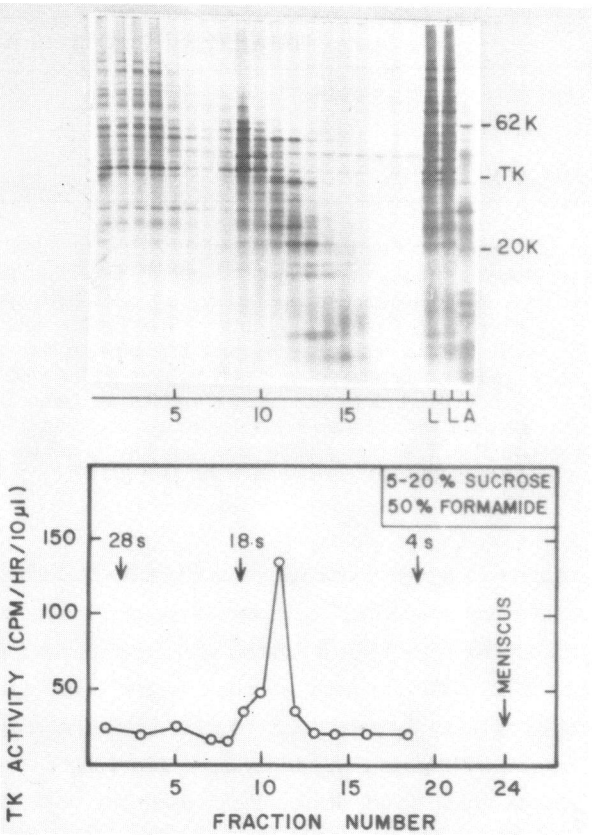

Figure 5: Velocity sedimentation of cytoplasmic RNA from HSV-infected cells. RNA was denatured in formamide and sedimented in sucrose with formamide as described in the text. RNA recovered from each fraction was used to program the synthesis of ^{35}S-methionine labeled proteins which were analyzed by polyacrylamide gel electrophoresis (10% acrylamide, 0.27% bisacrylamide, 7.5 M urea) and also the synthesis of enzymatically active HSV-TK. The position of 28s, 18s and 4s RNAs are indicated. Recovery of TK mRNA activity in the gradient was 90 percent of that applied to the gradient. The TK polypeptide is indicated on the electropherogram. Syntheses were carried out with unfractionated HSV-infected cell RNA (L) and with adenovirus-2 infected-cell RNA (A) and are indicated on the electropherogram. Reactions were carried out a 30°C and with 0.5 mM Mg.

enzyme is composed of two identical subunits which are coded by the TK gene of HSV. If we assume that the TK has an average amino acid composition, we can calculate that the TK protein has about 365 amino acids (42,000 ÷ 115 daltons/amino acid). This means that the protein requires an mRNA of 1100 nucleotides. The estimate of 1400 residues for the actual size of the mRNA from the sedimentation velocity agrees very well with the

expected size and allows for about 300 nucleotides of poly A and untranslated leader sequences. The fact that the measured size is near the minimal expected size argues that little or no aggregation of the TK mRNA occurred under the conditions of sedimentation which we employed. It is interesting to note that if the active enzyme is a dimer, assembly of active dimers from the synthesized subunits must occur *in vitro*.

The study of the post-transcriptional metabolism of HSV mRNA shows that bulk HSV-mRNA is polyadenylated and that HSV-specific nuclear RNA is larger than HSV-specific cytoplasmic RNA (18,19). Since TK mRNA can be retained on oligo dT cellulose columns, we conclude that this specific mRNA is similar to bulk HSV-mRNA in that it contains poly A at the 3' end. The size and translatability of nuclear transcripts of the TK gene are as yet unknown.

The availability of a simple translation system which makes active enzyme, the ease of genetic manipulation of the TK locus (15), and the physical isolation of the TK gene (20,21) all make the HSV-TK system attractive for study of RNA metabolism in mammalian cells.

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