Multiple mRNA Species for Adenovirus Type 2 Polypeptides III and pVII

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Fractionation of messenger activities isolated from the cytoplasm of HeLa cells late in infection with adenovirus type 2 reveals that viral polypeptides III and pVII are each synthesized from two different-sized mRNA's. The major messenger activity for each protein has the same sedimentation rate as that previously reported by Anderson et al. (Proc. Natl. Acad. Sci. U.S.A. 71:2756-2760, 1974). The minor messenger activities for III and pVII sediment more rapidly and are not aggregates of the major mRNA's for these proteins. The two minor messenger activities cosediment with two polyadenylated RNA species which are labeled late in infection with ^{32}P and whose molecular weights are estimated to be 2.9×10^6 and 2.4×10^6 . Both of these species hybridize to adenovirus type 2 DNA specific for the mRNA family that is 3' coterminal at adenovirus type 2 map position 49.5 and the mRNA family that is 3' coterminal at 62.0. This is consistent with the possibility that these RNAs have 5'-terminal sequences identical to those of the normal mRNA's for III and pVII but are 3' coterminal at map position 62, the normal 3' terminus of the mRNA's for polypeptides II and pVI. These species are not found in polyadenylated RNA isolated from the nucleus, suggesting that the minor mRNA species are cytoplasmic RNAs.

The late mRNA's of adenovirus type 2 (Ad2) coded to the right of Ad2 map position 16 are arranged in five families whose members have overlapping 3'-terminal sequences and different lengths of sequences in the 5' regions (6, 16, 19, 24). These mRNA's are synthesized by differential processing of a single unique transcript which is initiated at position 16 and transcribed rightward to the end of the genome (7, 8, 23, 25). Cleavage and polyadenylation of the nascent transcript occur at one of five possible sites which correspond to the 3' termini of the five mRNA families (20). The partially processed polyadenylated transcript is then further processed by the splicing of sequences at 16, 19, and 27 to form a 200-base 5' leader sequence (3, 5, 10) which is subsequently spliced to one of a limited number of acceptor sites coded within the family that lies to the 5' side of the polyadenylation site. The slicing of the leader to the main body of the mature mRNA may occur by way of a series of intermediates in which the leader is transferred to various acceptor sites along the transcript between the leader and the main body of the mRNA (18, 20).

In the course of fractionating cytoplasmic RNAs isolated from HeLa cells during the late phase of Ad2 infection, we observed two size classes of messenger activities for each of the viral proteins III and pVII. The major messenger

activities corresponded in size to those previously reported for these proteins. The minor messenger activities for these proteins were, however, much larger and may correspond to molecules which have a 3' terminus at the site normally present in the mRNA family immediately downstream from the family that encodes III and pVII.

MATERIALS AND METHODS

Cells. HeLa S3 cells were grown in suspension in Joklik-modified Eagle minimal essential medium supplemented with 5% calf serum.

Virus. Stocks of Ad2 were obtained from Gernot Walter. Cells were infected at a multiplicity of 20 PFU/cell. Virus was adsorbed for 30 min at room temperature and at a concentration of 4×10^7 cells per ml. Cells were then diluted to a concentration of 4×10^5 /ml with fresh medium, and infection was allowed to continue for 24 h.

Labeling of cells. Cells were labeled at 24 h after infection with $^{32}\text{P}_i$ (ICN Pharmaceuticals, carrier free). Infected cells (100 ml) were collected by centrifugation at 1,000 rpm and washed once with Eagle minimal essential medium without phosphate. Cells were resuspended in 50 ml of this phosphate-free medium supplemented with 2% dialyzed calf serum; 25 mCi of $^{32}\text{P}_i$ was added, and the cells were incubated for 3 h at 37°C.

Preparation of RNA. At 24 h after infection with Ad2, 4×10^7 HeLa cells were collected by centrifugation at 1,000 rpm, washed once with cold saline, and

suspended in 1.7 ml of cold isotonic lysis buffer (0.15 M NH_4Cl , 10 mM Tris [pH 6.8], 2 mM MgCl_2 , 50 μg of dextran sulfate per ml). Then 0.2 ml of 10% Nonidet P-40 (Shell Chemical Co.) was added, followed by brief blending in a Vortex mixer; 0.1 ml of 10% deoxycholate was added, and the suspension was blended for 30 s. Nuclei were pelleted by centrifugation at 2,000 rpm for 2 min, and then 90 μl of 0.5 M EDTA (pH 7.0), 80 μl of 4 M NaCl, 0.2 ml of 10% sodium dodecyl sulfate, and 20 μl of β -mercaptoethanol were added to the supernatant. To make cytoplasmic RNA, the supernatant was warmed to 30°C and extracted with 2 ml of phenol. Chloroform (2 ml) was added, and the mixture was reextracted. Phases were separated by centrifugation at 10,000 rpm for 20 min at 25°C. The aqueous phase was reextracted with 2 ml of chloroform, and the phases were separated as above. The aqueous phase was then collected, and RNA was precipitated by the addition of 2 volumes of ethanol. Precipitated RNA was stored in aqueous ethanol at -20°C. Polyadenylic acid [poly(A)]-containing cytoplasmic RNA was obtained by chromatography on oligodeoxythymidylic acid-cellulose (T-3; Collaborative Research, Inc.).

To make nuclear RNA, nuclear pellets were suspended in 1.0 ml of 0.5 M NaCl-50 mM MgCl_2 -0.01 M Tris (pH 7.5), 60 μl of 0.5 M EDTA, 50 μl of 20% sodium dodecyl sulfate, and 100 μl of proteinase K (5 mg/ml) were added, and the suspension was incubated at room temperature for 15 min. Water (1.0 ml) and β -mercaptoethanol (20 μl) were added, and the suspension was agitated briefly. The suspension was extracted with phenol and chloroform as described above for cytoplasmic RNA.

Poly(A)-containing nuclear RNA was selected by mixing the aqueous phase from the extracted nuclei with an equal volume of 0.5 M LiCl, 1 mM EDTA, and 10 mM Tris (pH 7.5). Oligodeoxythymidylic acid-cellulose (0.2 g) was added and kept in suspension by intermittent agitation at 4°C for 30 min, then collected by low-speed centrifugation and washed three times with 5 ml of ice-cold 0.5 M LiCl-1 mM EDTA-10 mM Tris (pH 7.5). Bound RNA was eluted by washing the oligodeoxythymidylic acid-cellulose two times with 1.0 ml of sterile water at room temperature. The eluted RNA was adjusted to 0.2 M sodium acetate (pH 5.2) and precipitated with 2 volumes of ethanol.

Sucrose gradient centrifugation. Nuclear or cytoplasmic RNAs in a total volume of 0.5 ml were layered on 10.6-ml gradients of 10 to 30% sucrose (wt/wt) in 10 mM sodium acetate (pH 5.2)-1 mM EDTA-0.1% sodium dodecyl sulfate. Centrifugation was performed at 20°C for 4.5 h at 40,000 rpm for nuclear RNAs and for 15 h at 30,000 rpm for cytoplasmic RNAs in a Beckman SW41 rotor.

Agarose gel electrophoresis. [^{32}P]RNA was reacted with glyoxal according to the procedure of McMaster and Carmichael (17). After reaction with glyoxal, the various samples were mixed with an equal volume of 1 mM EDTA (pH 7.0)-50% glycerol-0.001% bromophenol blue and applied directly to a 4-mm-thick 1% agarose gel (Sigma Type V) containing Leaning buffer E plus 0.1% sodium dodecyl sulfate. Electrophoresis was for 18 h at 75 V. After electrophoresis,

the gels were dried onto Whatman 3 MM paper. Radioactive RNA species were visualized by autoradiography enhanced with Du Pont Cronex Lightning-Plus intensifying screens.

Cell-free translation. RNAs were translated in the messenger-dependent rabbit reticulocyte lysate system described by Pelham and Jackson (21) in the presence of [^{35}S]methionine (Amersham Corp., 1,000 Ci/mmol).

Polyacrylamide gel electrophoresis. Proteins synthesized *in vitro* were resolved by diluting reaction mixtures with 5 volumes of sample buffer and applying 5 μl to each lane of a 1-mm-thick, 9.5-cm-long polyacrylamide slab gel according to the system described by Laemmli and Maizel (12, 15). Electrophoresis was for 4.5 h at a constant current of 15 mA.

DNA-paper and hybridization protocols. Purified plasmid DNA was coupled to diazobenzoyloxymethyl-paper by the method of Stark and Williams (22). Each coupling reaction contained 100 μg of sheared, denatured plasmid DNA and a 1.0-cm-diameter paper circle.

Hybridizations were performed in 50% formamide-0.4 M NaCl-20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.4)-5 mM EDTA-0.2% sodium dodecyl sulfate at 37°C for 5 h. Each reaction contained one DNA-paper circle, 200 μg of yeast tRNA, and 100,000 to 500,000 dpm of ^{32}P -labeled poly(A)-containing RNA in a final volume of 140 μl . The DNA-paper was washed three times (15 min each time) in 50% formamide-0.1 M NaCl-10 mM PIPES (pH 6.4)-5 mM EDTA-0.2% sodium dodecyl sulfate at 32°C, and two times (5 min each time) in 0.2 M NaCl-20 mM PIPES (pH 6.4)-5 mM EDTA-0.1% sodium dodecyl sulfate at 55°C. Hybridized RNA was eluted by washing the DNA-paper two times with 100 μl of 99% formamide-1 mM EDTA at 55°C and one time with 200 μl of 1.0 M EDTA at 55°C. Eluted RNA was precipitated from the pooled washes by the addition of 20 μg of yeast tRNA, 40 μl of 2 M sodium acetate (pH 5.2) and 1 ml of ethanol at -20°C.

RESULTS

Two messenger activities for III and pVII. Total cytoplasmic RNA was isolated from Ad2-infected HeLa cells at 24 h after infection and fractionated on a sucrose gradient. A portion of each fraction was translated in the messenger-dependent rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and the products were analyzed by polyacrylamide gel electrophoresis. A fluorogram of this gel is shown in Fig. 1. The major activities for III and pVII sedimented at 25S and 21S, respectively, as previously reported by Anderson et al. (1). Minor messenger activities for III and pVII were also found, sedimenting at 32S and 30S, respectively (Fig. 1, fractions 5, 6, and 7).

To examine the possibility that these represent aggregated forms of the major messenger activities, ^{32}P -labeled, poly(A)-containing RNA

FIG. 1. Translation of size-fractionated Ad2 mRNA's. A 100- μ g sample of poly(A)-containing RNA isolated from the cytoplasm of HeLa cells infected for 24 h with Ad2 was fractionated on sucrose gradients as described in the text. The gradient was divided into 0.45-ml fractions, and RNA in each fraction was collected by ethanol precipitation and then dissolved in 50 μ l of water; 1 μ l of each was translated in a 10- μ l reaction mixture as described in the text. One-tenth of each reaction mixture was analyzed on a 12.5% polyacrylamide gel, and 35 S-labeled proteins were visualized by fluorography.

was isolated from HeLa cells at 24 h after infection with Ad2. The RNA was sedimented through a sucrose gradient, and a portion of each fraction was translated and analyzed as shown in Fig. 1. The faster-sedimenting messenger activities for III and pVII were pooled, as well as the major peak of activity for III. The two RNA pools were precipitated, dissolved in buffer, and heat denatured; each was sedimented through a second gradient. The peak fractions of radioactivity from each gradient were again collected and sedimented through a third gradient as described above. The radioactivity profiles from the third gradient are shown in Fig. 2. [32 P]RNA associated with the fast-sedimenting messenger activities for III and pVII (Fig. 2A) still sedimented faster than did the [32 P]RNA associated with the major III messenger activity (Fig. 2B).

The RNAs in the peak fractions from each gradient were precipitated, and a sample of each was translated in vitro in the presence of [35 S]methionine and analyzed on a polyacrylamide gel. The translation products are shown in Fig. 3. The peaks of minor messenger activities for III and pVII (Fig. 3A) and the major peak of III mRNA activity (Fig. 3B) cosedimented with their respective peaks of radioactivity (Fig. 2A and B). The minor messenger activity for III sedimented slightly faster than did the minor messenger activity for pVII, suggesting the presence of two distinct RNA species. It was apparent that the minor messenger activities for III and pVII still sedimented more rapidly than did the major activity for III, even after 3 cycles of heat denaturation and sedimentation.

FIG. 2. Sedimentation of ^{32}P -labeled Ad2 mRNA's after three cycles of sedimentation and heat denaturation. ^{32}P -labeled, polyadenylated RNA was isolated as described in the text. Minor mRNA's (30S and 32S) for polypeptides III and pVII (A) and the major mRNA for III (B) were isolated from a sucrose gradient and subjected to two subsequent cycles of heat denaturation and sedimentation. The figure shows radioactivity present in each fraction of the third sucrose gradient.

To confirm that the rapidly sedimenting activities for III and pVII were not aggregates of smaller RNAs, the peak fraction of radioactivity from each gradient was treated with glyoxal (17) and analyzed on an agarose gel. The peak fraction of radioactivity from Fig. 2A (which has messenger activity for both III and pVII) contained two species of RNA (Fig. 4, lanes 1 and 4) designated *a* and *b*. It has recently been demonstrated that the only polyadenylated RNAs transported to the cytoplasm late in infection are adenovirus specific (2). It is demonstrated below that the ^{32}P -labeled species *a* and *b* are cytoplasmic and contain sequences from the mRNA family located at map positions 39.0 to 49.5, strongly suggesting that they do in fact correspond to the minor mRNA's for polypeptides III and pVII. Since the minor mRNA for III sedimented slightly faster than did the minor mRNA for pVII, it is likely that the higher-

molecular-weight species *a* is the minor mRNA for III and that species *b* is the minor mRNA for pVII. The peak fraction from Fig. 2B (the major mRNA for III) contains three labeled bands (species *c*, *d*, and *e*), which migrated more rapidly than did species *a* and *b* (Fig. 4, lanes 2 and 5). Since the peak fraction from Fig. 2B is associated with messenger activities for the III, 100K, and pVI proteins (Fig. 3B) specified by

FIG. 3. Translation of RNAs isolated as described in the legend to Fig. 2. RNA in each fraction of the gradients in Fig. 2 was collected by ethanol precipitation and dissolved in 10 μl of water, 1 μl from each fraction was translated in vitro, and ^{35}S -labeled proteins were analyzed on a 10% polyacrylamide gel and visualized by fluorography, as described in the text. Fractions in (A) and (B) are the same as those from Fig. 2 (A) and (B), respectively.

FIG. 4. Agarose gel electrophoresis of [^{32}P]-mRNA's. ^{32}P -labeled RNAs were treated with glyoxal and analyzed on a 1.0% agarose gel as described in the text. Lane 1, Fraction 8 from Fig. 2A; lane 2, fraction 11 from Fig. 2B; lane 3, total ^{32}P -labeled, poly(A)-containing RNA from HeLa cells infected with Ad2 for 24 h; lanes 4 to 6, same as 1 to 3 except exposed longer; lane 7, [^{14}C]rRNA's; Lane 8, same as lanes 3 and 6.

adenovirus, it was not surprising to find three ^{32}P -labeled RNA species in this fraction. Total ^{32}P -labeled, poly(A)-containing, late Ad2 RNA was also run in a parallel lane (Fig. 4, lanes 3 and 6). Species *a*, *b*, *c*, and *d* are evident in this RNA. Species *f* and *g* are identified below as the mRNA for hexon and the 21S mRNA for pVII, respectively.

32S and 30S ^{32}P -labeled species have sequences from Ad2 map positions 41.0 to 50.1 and 56.5 to 63.7. To prove that the high-molecular-weight ^{32}P -labeled RNA species contained sequences from the region of the Ad2 genome coding for III and pVII, ^{32}P -labeled, cytoplasmic, poly(A)-containing RNA from Ad2-infected HeLa cells was hybridized to a cloned Ad2 *Hind*III D (map position 41.0 to

50.1) restriction fragment immobilized on diazobenzyloxymethyl-paper. Hybridized RNA was eluted, treated with glyoxal, and analyzed on an agarose gel. An autoradiograph of this gel is shown in Fig. 5. Two major ^{32}P -labeled species which migrated faster than did 28S RNA hybridized to this DNA (Fig. 5, lane 3). They most likely correspond to the major mRNA's for III and pVII. The minor species we have designated *a* and *b* also annealed specifically to *Hind*III D DNA, demonstrating the presence of sequences from map positions 41.0 to 50.1 in these species.

Because the radiolabeled species *a* and *b* cosedimented with the minor messenger activities for III and pVII and contained sequences from the region of the Ad2 genome known to code for these proteins, we conclude that these species are in fact the minor mRNA's for these two proteins. Since only 5'-terminal genes on potentially polycistronic eucaryotic mRNA's are translated (11), it follows that the coding sequences for III and pVII are likely to be on the 5' terminus of species *a* and *b* and that additional sequences not normally present in III and pVII mRNA's are present on their 3' termini. One possibility is that these additional sequences are coded by a region of the Ad2 genome immediately downstream from the III and pVII mRNA family, namely, the region which normally specifies II and pVI mRNA's. To test this hypothesis, ^{32}P -labeled, cytoplasmic, poly(A)-containing RNA isolated from Ad2-infected HeLa cells was

FIG. 5. Selection of ^{32}P -labeled cytoplasmic RNAs on restriction fragments of Ad2 DNA. ^{32}P -labeled cytoplasmic, poly(A)-containing RNA was hybridized to cloned Ad2 restriction fragments as described in the text. Selected RNA was treated with glyoxal and analyzed on a 1.2% agarose gel. Lane 1, [^{14}C]rRNA; lane 2, total ^{32}P -labeled, cytoplasmic poly(A)-containing RNA isolated from HeLa cells infected with Ad2 for 24 h; lane 3, ^{32}P -labeled RNA hybridizing to cloned Ad2 *Hind*III D DNA; lane 4, ^{32}P -labeled RNA hybridizing to cloned Ad2 *Pst*E DNA; lane 5, same as lane 4 except longer exposure of autoradiograph.

hybridized to a cloned Ad2 *Pst*E (map position 56.5 to 63.7) restriction fragment immobilized on diazobenzylxymethyl-paper. Two major species of RNA hybridized to this DNA fragment (Fig. 5, lanes 4 and 5), corresponding to the mRNA's for pVI and II. Both species *a* and *b* also hybridized to this DNA, demonstrating the presence of sequences coded in the 56.5 to 63.7 region in these RNAs. Species *a* and *b* do not hybridize to DNA specific for fiber mRNA (data not shown).

The 32S and 30S mRNA's are not found in the nucleus. The presence of high-molecular-weight messenger activities for III and pVII among cytoplasmic mRNA's suggested that some partially processed nuclear RNAs may have been present in our cytoplasmic mRNA preparations. To determine whether species *a* and *b* were present in the nucleus, ³²P-labeled, nuclear, poly(A)-containing RNA was isolated and sedimented through a sucrose gradient. A sample of each fraction in the 20S-to-45S size range was treated with glyoxal and analyzed on an agarose gel. Figure 6 shows an autoradiograph of this gel. A number of prominent labeled bands can be resolved; however, none of them comigrated with bands *a* and *b*. One prominent nuclear species migrated very close to band *a* but was consistently found to migrate just slower than *a* in a number of different experiments. No nuclear RNA species comigrated with band *b*. This experiment demonstrates that the presence of species *a* and *b* in the cytoplasmic RNA is not due to contamination of our cytoplasmic preparations with nuclear RNA. The possibility that small amounts of species *a* and *b* are present in the nucleus, however, cannot be excluded.

DISCUSSION

The major messenger activities for III and pVII have previously been shown to sediment at approximately 25S and 21S, respectively (1). These mRNA's have been mapped on the Ad2 genome to the mRNA family which has a common 3' terminus at about map position 49.5 (4, 6, 13, 14). The 5' ends of the main body of the mRNA's for III and pVII are probably located at 38.8 and 42.8, respectively (4, 6). These map coordinates are consistent with the observed size of the mRNA's from these proteins as determined by sedimentation (Fig. 1) and migration on gels (Fig. 4). Thus, these two mRNA's overlap in sequence, with III mRNA having additional sequences present on the 5' portion of the molecule which are likely to be those that actually code for the III polypeptide. The sequences that code for pVII are present internally in III mRNA

but are not translated.

The present paper describes two minor mRNA's coding for III and pVII which are considerably greater in size than the major mRNA species for these proteins. Assuming that the coding region for these proteins is at the 5' end of these large molecules, then the additional sequences must be at the 3' end of the molecule. The large messenger activities cosedimented with two ³²P-labeled cytoplasmic poly(A)-containing RNA species (designated *a* and *b*) which hybridize to a region of the Ad2 genome specific for the III and pVII mRNA family and also to a region specific for the II and pVI mRNA family. The molecular weights of *a* and *b* have been estimated by comparison of the migration of glyoxal-treated species with the migration of glyoxal-treated 18S and 28S rRNA's (Fig. 4, lanes 7 and 9). The molecular weights of *a* and *b* were thus determined to be about 3.3×10^6 and 2.7×10^6 , respectively. Using the same standards, the molecular weight of hexon mRNA (species *f*) was estimated to be about 1.5×10^6 . However, the heteroduplex analyses of Chow et al. (4, 6) indicate that hexon mRNA spans about 10.5 map units of the Ad2 genome (molecular weight, 23×10^6) (9), which would correspond to a molecular weight of about 1.3×10^6 , including leader sequence and poly(A). Thus, use of 18S and 28S rRNA as molecular weight standards in this gel system apparently results in a small overestimation of the molecular weight of hexon mRNA. If we have overestimated the size of *a* and *b* by a similar proportion, their actual molecular weights would be 2.9×10^6 and 2.4×10^6 and would span 24 and 20 map units of the genome, respectively. Therefore, these molecules are each approximately 10.7 map units longer than the major mRNA's for III and pVII (1, 4, 6). This is quite close to the length of the mRNA family that lies immediately downstream from the III and pVII family. Since *a* and *b* are composed in part of sequences from this downstream region, it is likely that these RNA species have 5' sequences that are identical to those of the normal mRNA's from III and pVII that are 3' coterminal with the mRNA's for pVI and II (hexon) at map position 62 instead of the normal 3' terminus for the III and pVII mRNA's at 49.5.

How might such molecules arise? The majority of molecules with 3' termini coded at 62 are destined to become mRNA's for pVI or II (4, 6, 20). Thus, it is likely that the large mRNA's for III and pVII arise by incomplete or aberrant processing of transcripts which normally would produce pVI or II mRNA's. Processing of these transcripts would normally involve the complete

FIG. 6. Analysis of 20S to 45S ^{32}P -labeled, nuclear, poly(A)-containing RNAs on an agarose gel. ^{32}P -labeled, nuclear, poly(A)-containing RNA was fractionated on a sucrose gradient. RNA in the 20S to 45S region of the gradient (fractions 8 to 20 as indicated) and total poly(A)-containing cytoplasmic RNA were treated with glyoxal and analyzed on a 1.0% agarose gel as described in the text.

removal of sequences between the 3' end of the leader sequence at map position 27 and the 5' end of the body of pVI mRNA at 49.5. The processing of the fiber mRNA precursor apparently involves the removal of RNA sequences coded between the leader and main body of the mRNA in a series of steps (18), which may involve a transfer of the 5' leader to various

acceptor sites along the length of the precursor. If the processing of the mRNA precursor for II and pVI mRNA's occurs in steps in which the 3' end of the leader is first spliced to 5'-mRNA sequences in the family which normally codes for III and pVII and then is subsequently spliced to 49.5 (pVI mRNA) or 51.2 (II mRNA), then the large mRNA's for III and pVII might be

intermediates in this pathway which are transported to the cytoplasm before completion of processing.

Alternatively, if removal of sequences between map positions 27 and 49.5 or 51.2 occurs normally in a single step, then the large mRNA's for III and pVII could arise by a processing step in which sequences at map position 27 are spliced to sequences at 38.8 (III mRNA) or 42.8 (pVII mRNA). This would be an abnormal splice for a transcript with a 3' terminus at 62 but an entirely normal splice for molecules which have 3' termini at 49.5. In this event, the large mRNA's for III and pVII would be formed from an abnormal processing event of a precursor to pVI or II mRNA resulting in a large mRNA for III or pVII. Such a molecule may not be further processed because an appropriate nucleotide sequence or RNA secondary structure which would allow the leader to be spliced again to map position 49.5 or 51.2 may not be present once the leader has been spliced to 38.8 or 42.8.

Further analysis of the structure of intermediates in the synthesis of pVI and II mRNAs should distinguish between these two possibilities. In the event that species *a* and *b* prove to be normal intermediates in the synthesis of these mRNA's, they may be useful as substrates for studying the biochemistry of mRNA processing because they are readily isolated from cytoplasmic extracts of infected cells.

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