Adenovirus Type 2 Fiber mRNA Synthesis: No Evidence for a Cytoplasmic Processing Pathway

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Adenovirus type 2 fiber mRNA exists in several forms in the cytoplasm which differ in the presence or absence of extra 5'-leader segments (L. T. Chow and T. R. Broker, *Cell* 15:497-510, 1978). We have investigated the possibility that forms possessing extra leader segments serve as precursors to the mature form in the cytoplasm. Pulse-labeled fiber mRNA became considerably shorter (150 to 250 bases) during a chase; however, most of the pulse-labeled species failed to hybridize to DNA fragments known to encode extra leader segments. Moreover, the entire decrease in size appeared to be due to extensive shortening of the polyadenylic acid tail. Mature-sized fiber mRNA was synthesized normally in cells infected with the nondefective adenovirus type 2-simian virus 40 hybrid virus Ad2⁺ND5, in which the region encoding the extra leader segments is deleted. These results indicate that the additional 5'-leader segments present in wild-type adenovirus type 2 fiber mRNA are not required for the production of mature fiber mRNA and that species that possess them are not cytoplasmic precursors to the mature form.

The late phase of adenovirus type 2 (Ad2) infection of HeLa cells is a convenient system in which to study the regulation and biochemistry of mRNA biogenesis. Most of the viral RNA synthesized in this period is transcribed from a single large transcription unit approximately 30 kilobases in length (17), which has a cap site at 16.4 map units (m.u.) (39) and a termination site at approximately 98 m.u. (14) on the Ad2 genome. The primary transcript of this large transcription unit is unique among eucaryotic mRNA precursors in that it is processed by a number of different pathways to produce at least 14 different mRNA species (8, 11, 27, 31). These mRNAs can be grouped into five families whose members have overlapping sequences and share common 3' termini (15, 31, 40). All of the mRNAs specified by this transcription unit are spliced molecules composed of a 5'-terminal untranslated leader sequence and a main body which specifies a particular protein (5, 10, 16, 22). The same leader sequence is present on all of these mRNAs and is composed of three spliced segments coded in the virus genome at 16.4 m.u. (41 bases), 19.6 m.u. (72 bases), and 26.6 m.u. (90 bases) (2, 3, 37, 38).

The detailed pathways by which the primary transcript of this transcription unit is processed are not known. Early events in the processing pathway are (i) cleavage and polyadenylation of the primary transcript at one of five possible sites which correspond to the 3' termini of the

five mRNA families (32) and (ii) methylation of internal adenosine residues in sequences destined to be conserved in the mature mRNA (7). Further processing involves splicing together of the three segments of the 5' leader and the splicing of these leader segments to the body of the mature mRNA. Apparently several of these splicing events do not necessarily occur in single steps but through a series of intermediate structures in which portions of intervening sequences are removed. Several intermediate species have been detected in nuclear RNA from Ad2-infected cells (6). Intermediates in the processing of fiber mRNA have also been demonstrated in nuclear RNA which are likely to represent the partial removal of the intervening sequences between the 5'-leader segments and the main body of fiber mRNA (30). These various processing steps involved in maturation of the primary transcript are thought to occur in the nucleus.

Several studies have demonstrated that some of the Ad2 late mRNAs present in the cytoplasm contain leader segments in addition to those normally present in the 5' leader. Certain Ad2, Ad3, and Ad7 late mRNAs can have an extra segment located between the usual second and third leader segments (9, 20, 21). The mRNA for polypeptide IV (fiber) can have additional segments between the 5' leader and the main body. The most frequently observed is 81 bases long and is coded at 78.5 m.u. (8, 38). This segment

has no AUG codon, and its presence has no effect on the ability of the mRNA to be translated *in vitro* (12). Chow and Broker (8) and Kilpatrick et al. (20) have suggested that molecules that contain extra leader segments may be authentic precursors to mature mRNA species, and because such molecules have been isolated with polyribosomes it is possible that the subsequent processing of these precursors occurs in the cytoplasm. However, no kinetic evidence supporting this concept has been reported.

We have investigated the possibility that fiber mRNA heterogeneity in the cytoplasm could be due to the existence of such a precursor-product relationship between the multiple cytoplasmic species containing extra leader segments and the predominant form of fiber mRNA detected in the cytoplasm of Ad2-infected HeLa cells. We present kinetic and structural data which render this possibility highly unlikely for the metabolism of fiber mRNA.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were grown in suspension culture and infected with Ad2 or the Ad2-simian virus 40 (SV40) hybrid virus Ad2⁺ND5 as previously described (24). All experiments were initiated with cells infected for 24 h.

Radiolabeling of RNA. Infected cells were labeled with ³²P_i (New England Nuclear Corp.) as previously described (24). RNA was isolated from cells labeled for 2 or 3 h as indicated (pulse) or from cells labeled for 3 h in phosphate-deficient medium followed by incubation in normal phosphate-containing medium for an additional 8 h (chase).

[³H]uridine pulse labels and glucosamine-unlabeled-uridine chase experiments were performed by a modification of the protocol of Levis and Penman (25). Glucosamine (Sigma) (pH 7.4) was added to 100 ml of infected cells at a concentration of 20 mM for 30 min. Cells were collected by low-speed centrifugation, suspended in 100 ml of fresh medium containing 50 to 100 μ Ci of [5,6-³H]uridine (Amersham) per ml, and incubated for 60 min at 37°C. The chase was initiated by the addition of glucosamine to 20 mM and uridine and cytidine (Sigma) to 5 mM. To reduce glucosamine toxicity, cells were collected after 30 min and suspended in an equal volume of fresh medium containing 5 mM each of uridine and cytidine. There was no significant increase in radioactivity in polyadenylic acid [poly(A)]-containing RNA after initiation of the chase.

Isolation of RNA. Poly(A)-containing cytoplasmic RNA was isolated as previously described (24).

To make total cellular poly(A)-containing RNA, 4×10^6 cells were harvested, washed once in ice-cold saline, and suspended at room temperature in 2.0 ml of 7 M urea–2% sodium dodecyl sulfate (SDS)–0.35 M NaCl–2 mM EDTA–10 mM Tris (pH 7.8). DNA was sheared by repeated passage through a 21-gauge needle. The suspension was then extracted with phenol and chloroform as previously described (24), and nucleic acids were precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol

(–20°C). Precipitated nucleic acids were dissolved in 5 ml of water, salt concentrations were adjusted to 10 mM sodium acetate (pH 5.2)–0.5 M LiCl–1 mM EDTA–0.1% SDS, and poly(A)-containing RNA was isolated by batchwise selection with 0.1 g of oligodeoxythymidylic acid-cellulose (Collaborative Research). The cellulose was washed extensively with the above buffer, and bound RNA was eluted with 1.5 ml of water and precipitated by the addition of 0.2 ml of 2 M sodium acetate (pH 5.2) and 5 ml of ethanol.

Hybridization of RNA to immobilized DNA. Plasmids possessing various *Sma* and *EcoRI* fragments of Ad2 DNA were constructed in this laboratory by insertion into the *Pst* site of pBR322. Plasmids possessing *Bal*, *HindIII*, *Pst*, or *Bam* fragments of Ad2 DNA were the generous gift of Sue Berget. The plasmid pJJ1, containing the entire SV40 genome, was the gift of John Wilson.

Purified plasmid DNA was coupled to diazobenzyl-oxy-methyl-paper by the method of Stark and Williams (34). Each coupling reaction contained 100 μ g 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% SDS, and 2 mg of yeast tRNA per ml at 42°C for 6 to 16 h. RNA to be hybridized was dissolved in the above buffer in a total volume of 10 μ l and applied to a prewashed DNA-paper circle which had been blotted to remove excess buffer and placed in a siliconized glass vial. After hybridization, DNA-paper circles were washed four times for 5 min each with 3 ml of 0.2 M NaCl–10 mM PIPES (pH 6.4)–5 mM EDTA–0.1% SDS at 60°C. RNA was eluted by heating the filter to 90°C in 0.45 ml of 1 mM EDTA (pH 7.0) and precipitated by the addition of 20 μ g of yeast tRNA, 50 μ l of 0.2 M sodium acetate (pH 5.2), and 1 ml of ethanol.

Gel electrophoresis of RNA. All labeled RNAs analyzed were denatured before electrophoresis with glyoxal by the procedure of McMaster and Carmichael (28).

³H- and ³²P-labeled poly(A)-containing RNAs were resolved on horizontal 4-mm-thick 1.2% agarose (Sigma type V) gels in Loening buffer E (26). Electrophoresis was for 16 h at 2 to 3 V/cm. ³²P-leader segments were resolved on 15% acrylamide gels by discontinuous gel electrophoresis as described by Laemmli (23).

³²P-labeled RNA species were visualized as previously described (24). [³H]RNA was visualized by preparing agarose gels for fluorography as follows. The agarose gel was immersed in two changes of methanol for 2 h each at 37°C, then in 5% (wt/vol) 2,5-diphenyloxazole dissolved in methanol for 4 h at 37°C, and then in water for 16 h at 37°C. The gel was then dried onto Whatman 3MM paper.

Removal of poly(A) tracts. Poly(A) was removed from ³²P-labeled mRNAs by digesting the RNA with RNase H in the presence of polydeoxythymidylic acid, using a modification of the procedure of Harris (18). Each 10- μ l digestion contained approximately 5,000 cpm of [³²P]RNA, 10 μ g of unlabeled tRNA, 1 μ g of polydeoxythymidylic acid (Collaborative Research), and 0.6 U of *Escherichia coli* RNase H (Enzo Biochem) in 0.05 M Tris (pH 7.5)–0.01 M magnesium acetate–0.001 M EDTA–0.01 M dithiothreitol–10% glycerol. The samples were digested for 30 min at

FIG. 1. Size analysis of Ad2 mRNAs at short and long times after their appearance in the cytoplasm. Poly(A)-containing total cytoplasmic RNA was isolated from HeLa cells infected for 24 h with Ad2 and pulse-labeled for 2 h with ^{32}P (lanes P) or pulse-labeled for 2 h and then chased for 8 h (lanes C). Total poly(A)-containing [^{32}P]RNA and [^{32}P]RNA prepurified by hybridization to various cloned Ad2 restriction fragments were denatured with glyoxal and analyzed on a 1.2% agarose gel. Lanes on gel (left to right): ^{14}C -labeled HeLa cell rRNAs; total poly(A)-containing RNA; RNA selected by *Hind*III-D DNA; RNA selected by *Pst*-E DNA; RNA selected by *Eco*RI-D DNA; RNA selected by *Eco*RI-E DNA; [^{14}C]rRNA. Numbers indicate m.u. of DNA fragment.

30°C, and RNA was precipitated by the addition of 0.4 ml of 0.2 M sodium acetate (pH 5.2)–0.001 M EDTA–0.1% SDS and 1.0 ml of ethanol. Precipitated RNA was collected by centrifugation and denatured with glyoxal as described above.

RESULTS

Size changes in Ad2 late mRNAs in the cytoplasm. Cytoplasmic processing of an mRNA species would be indicated by a decrease in its size after its transport from the nucleus. We therefore compared the size of cytoplasmic Ad2 late mRNAs isolated after their initial appearance in the cytoplasm and several hours later. Cytoplasmic RNA was isolated from detergent-lysed HeLa cells infected with Ad2 and labeled with $^{32}\text{P}_i$. Under these conditions unprocessed high-molecular-weight RNA remained associated with nuclei, and only mRNA-size molecules were found in the cytoplasmic extract. RNA specific for various late mRNA families was isolated by hybridization to cloned Ad2 DNA restriction fragments immobilized on diazobenzylxymethyl-paper. RNAs were denatured with glyoxal, resolved on 1.2% agarose gels, and visualized by autoradiography.

Figure 1 shows a comparison of selected RNAs from four of the five 3'-coterminal mRNA families pulse-labeled for 2 h with ^{32}P (lanes labeled P) or pulse-labeled for 2 h and then

chased for an additional 8 h in phosphate-containing medium (lanes labeled C). A unique set of RNA species was selected by each family-specific DNA fragment. The size of the Ad2 RNAs corresponded well to that expected from heteroduplex analyses of Ad2 mRNAs (8, 11). Cell-free translation of RNA selected by these DNA fragments also resulted in the synthesis of the expected polypeptides.

Table 1 summarizes the size and probable identification of the RNA species detected in Fig. 1. RNA species isolated from ^{32}P -pulse-labeled cells migrated slower than the corresponding species isolated after a chase, indicating that each RNA species had been shortened by approximately 100 nucleotides with the exception of the mRNA for fiber (Fig. 1, *Eco*RI-E, lanes P and C), which decreased by approximately 150 to 250 nucleotides. Also, in contrast to the other mRNAs examined, fiber mRNA appeared in the cytoplasm as a heterogeneous species in pulse-labeled RNA but as two discrete bands after the 8-h chase period (see Fig. 1, 2, and 3). The faster migrating of these two species is likely to be the form of fiber mRNA composed solely of the 5'-tripartite leader and the main body of the fiber gene. The slower migrating of the two is likely to be the form possessing a fourth leader segment encoded at 78.5 m.u. and which has been previously reported and sequenced (8, 38). These proposed structures were confirmed in later experiments.

TABLE 1. Size of selected Ad2 cytoplasmic RNAs

DNA fragment used for hybridization (m.u.)	Approximate size of annealed RNA species ^a (nucleotides)		Approximate change in size (nucleotides)	Probable polypeptide coding assignment
	Pulse	Chase		
40.9–50.1	3,700	3,600	100	III
	2,400	2,300	100	pVII
	1,850	1,750	100	V
56.8–64.5	4,300	4,200	100	pVI
	3,600	3,500	100	II
75.9–83.4	4,200	4,100	100	100K
	2,300	2,200	100	pVIII
	2,050	1,950	100	
	1,800	1,700	100	
83.4–89.7	2,150–2,300	2,000	150–300	IV

^a Estimated from the migration of RNA species observed in Fig. 1, relative to 28S and 18S rRNAs. HeLa 28S and 18S RNAs were taken to be 4,400 and 1,975 nucleotides, respectively; these values were determined by comparison of their migration with numerous DNA and RNA standards of known size in denaturing gels (John Rogers, personal communication).

FIG. 2. Size analysis of precursors to fiber mRNA isolated from total cellular RNA. Total nucleic acids were isolated from infected cells pulse-labeled for 1 h with [3 H]uridine or pulse-labeled for 1 h and then chased for various times in glucosamine plus unlabeled uridine. Fiber mRNA-specific species were selected by annealing to immobilized *Eco*RI-D DNA and then denatured with glyoxal and resolved on a 1.2% agarose gel. The figure shows a fluorograph of the gel. Lanes: (A) cytoplasmic fiber mRNA from cells pulse-labeled for 3 h with 32 P; (B) whole-cell fiber mRNA species from cells pulse-labeled for 1 h with [3 H]uridine; (C, D, E, F, and G) same as B except with 1, 2, 3, 5, and 8 h of chase, respectively; (H) same as (A) but with an 8-h chase.

The large decrease in size of fiber mRNA species compared to the other viral mRNAs is consistent with the possibility that mature fiber mRNA is generated in the cytoplasm by the splicing of species with additional leader segments. An alternative explanation for the apparent decrease in size of fiber mRNA in the cytoplasm is that the nuclear processing pathway that gives rise to the mature form of fiber mRNA may be slower than the pathway that results in cytoplasmic fiber mRNAs with extra leader segments. Thus, the first species to appear in the cytoplasm would be those with extra leader segments, followed some time later by the appearance of the mature form, giving the illusion of a decrease in size of the cytoplasmic mRNA. If this were the case, then a prominent precursor to the mature form of fiber mRNA should be observed in the nucleus at the same time that the species possessing extra leader segments are present in the cytoplasm. To address this possibility we examined the kinetics of

appearance of label into fiber-related RNA species isolated from total cellular RNA during a [3 H]uridine pulse, glucosamine-uridine chase experiment. This labeling method was chosen to follow the appearance and fate of fiber mRNA precursors during an effective chase, which is not possible with 32 P_i as a label.

Infected cells were pulse-labeled for 1 h with [3 H]uridine and chased for various lengths of time with glucosamine and cold uridine. Poly(A)-containing fiber-specific RNAs were isolated from cellular nucleic acids and analyzed on a 1.2% agarose gel. A fluorograph of this gel is shown in Fig. 2. Fiber-specific RNA isolated after a 1-h pulse (lane B) consisted of a complex population of heterogeneous high-molecular-weight RNA species with a number of prominent discrete species also evident. The largest of these was approximately 25 kilobases in length, the predicted size of a primary transcript from 16.4 to 91.3 m.u. (the 3' terminus of fiber mRNA). A considerable portion of the radioactivity lay in a somewhat heterogeneous species which comigrated with the 32 P-labeled cytoplasmic fiber mRNA (lane A). After 1 h of chase (lane C), much of the high-molecular-weight heterogeneous fiber-specific RNA disappeared and most of the radioactivity comigrated with pulse-labeled cytoplasmic fiber mRNA (lane A). During subsequent chases of 2, 3, 5, and 8 h (lanes D, E, F, and G, respectively) the conversion of this heterogeneous species to two prominent discrete species was observed. The more abundant of these species comigrated with the mature form of 32 P-labeled cytoplasmic mRNA (lane H). (The less abundant and slower migrating of the two species is not reproduced well in the figure, but comigrated with the minor fiber mRNA species possessing an additional leader segment.) This experiment demonstrated that there is no precursor to the rapidly migrating fiber mRNA species present after an 8-h chase other than the cytoplasmic species labeled in a 1- to 2-h pulse.

Localization of regions on the Ad2 genome specifying leader segments on newly synthesized fiber mRNA. To identify regions of the Ad2 genome which specify extra leader segments on fiber mRNA, 32 P-labeled cytoplasmic fiber mRNA, either labeled for 3 h or labeled for 3 h and chased for 8 h, was isolated by preparative hybridization to cloned Ad2 *Eco*RI-E DNA immobilized on diazobenzoyloxymethyl-paper. The RNA was then hybridized a second time to various cloned Ad2 DNA fragments representing regions of the Ad2 genome between the promotor at 16.4 m.u. and the 5' terminus of the main body of the fiber gene at 86.3 m.u. RNA isolated after the second hybridization was analyzed on a 1.2% agarose gel. The two right-hand

FIG. 3. Identification of regions of the Ad2 genome specifying leader sequences present on fiber mRNA. ^{32}P -labeled cytoplasmic fiber mRNA from infected cells pulse-labeled for 3 h (lanes P) or pulse-labeled for 3 h and chased for an additional 8 h (lanes C) was isolated by preparative hybridization to cloned *EcoRI*-E DNA, immobilized on paper. This RNA was hybridized to other immobilized DNA fragments from different regions of the genome. Selected RNA was eluted, denatured with glyoxal, and analyzed on a 1.2% agarose gel. Lanes from left to right: ^{14}C -labeled HeLa cell rRNAs; ^{32}P -labeled fiber mRNA selected by *Bal*-E DNA, *Bal*-D DNA, *Bam*-D DNA, *Bam*-C DNA, *Sma*-A DNA, and *EcoRI*-D DNA; [^{32}P]RNA selected on *EcoRI*-E DNA, which was the starting material for the other hybridizations.

lanes in Fig. 3 (*EcoRI*-E) show ^{32}P -pulse-labeled fiber mRNA (lane P) and pulse-labeled and chased fiber mRNA (lane C), which was the starting material for the experiment. These species also hybridized to *Bal*-E DNA (14.7 to 21.5 m.u.) and *Bal*-D DNA (21.5 to 28.5 m.u.); this was expected because the first and second leader segments and third segment, respectively, are specified by DNA within these regions. No fiber mRNA-related sequences annealed to DNA fragments derived from 29.0 to 76.8 m.u. (*Bam*-D, *Bam*-C, and *Sma*-A). (The higher-molecular-weight species selected by these fragments are mRNAs which were originally present in the starting material as nonspecific contaminants.)

A considerable amount of pulse-labeled fiber mRNA annealed to DNA within the *EcoRI*-D fragment (75.0 to 84.0 m.u.); however, only the slower-migrating portion of the heterogeneous fiber mRNA species was selected (compare Fig. 3, *EcoRI*-E and *EcoRI*-D, lanes P). Likewise, the faster-migrating and more prominent species of the pulse-labeled and chased fiber mRNA did not hybridize to 75.9 to 84.0 m.u. DNA (compare Fig. 3 *EcoRI*-E and *EcoRI*-D, lanes C); however, the slower-migrating species did hybridize to this region. Thus, the major fraction of fiber mRNA labeled in a pulse or after a lengthy chase hybridizes only to sequences derived from DNA known to specify the main body of the mRNA or from DNA known to specify the three-segment leader sequence. A minor fraction of fiber mRNA is also composed of these

sequences plus an additional segment derived from between 75.9 and 84.0 m.u.

Size change of fiber mRNA in the cytoplasm is due to metabolism of poly(A). Because the major fraction of pulse-labeled fiber mRNA failed to hybridize to regions of the genome known to encode additional leader segments, we investigated the possibility that the large size change observed for fiber mRNA simply resulted from the metabolism of poly(A). This was done by analyzing the size of molecules from which poly(A) had been removed by annealing to poly-deoxythymidylic acid and digesting with RNase H. mRNA was isolated from cells pulse-labeled with ^{32}P or pulse-labeled and chased for 8 h in phosphate-containing medium. Fiber mRNA was isolated by hybridization to immobilized DNA from 84.0 to 89.7 m.u., and L4 mRNAs (100K and pVIII) were isolated by hybridization to immobilized DNA from 72.6 to 76.9 m.u. A sample of each mRNA was denatured and analyzed directly by gel electrophoresis, and a second sample was analyzed after removal of the poly(A) tail. Figure 4 shows an autoradiogram of the gel. The 100K and pVIII mRNAs decreased in size by approximately 100 nucleotides during the chase period, and fiber mRNA decreased in size by approximately 200 nucleotides as observed previously. However, after the removal of poly(A), all species migrated more rapidly, and there was no difference in the size of pulse-labeled or pulse-labeled and chased species, indicating that the entire decrease in

FIG. 4. Analysis of late region 4 mRNAs and fiber mRNA after removal of poly(A) tails. Cytoplasmic poly(A)-containing RNA was isolated from Ad2-infected HeLa cells pulse-labeled for 2 h with ^{32}P (lanes P) or pulse-labeled and then chased for 8 h (lanes C). Late region 4-specific RNA species (100K and pVIII mRNAs) were isolated by preparative hybridization to Ad2 72.6-to-76.9-m.u. DNA, and fiber mRNA was selected by hybridization to 83.4-to-89.7-m.u. DNA. RNAs were incubated with or without RNase H and polydeoxythymidylate to remove poly(A) tails, as described in the text, denatured with glyoxal, and analyzed on a 1.2% agarose gel.

size for the late mRNAs is due to the metabolism of poly(A). In particular, there was essentially no difference in the patterns of fiber mRNA species after removal of poly(A). Thus, the large decrease in size of fiber mRNA in the cytoplasm is apparently due to a more rapid metabolism of poly(A) than in the other late mRNAs.

Synthesis of fiber mRNA in cells infected with the Ad2-SV40 hybrid virus Ad2⁺ND5. Ad2⁺ND5 is a nondefective hybrid virus whose genome consists of Ad2 DNA with a deletion of the 78.7 to 85.5 m.u. region and an insertion of a portion of the early region of SV40 from 0.39 to 0.11 m.u. (19). Included in the deleted sequences is the region of Ad2 DNA which encodes the major extra ("y") leader segment which is seen in steady-state fiber mRNA isolated from cells infected with the wild-type virus. Zain et al. (38) have shown that the predominant form of fiber mRNA present in cells infected with this virus has no extra leader segment, demonstrating that an intermediate possessing the y segment is not required for the synthesis of the mature form. However, it is possible that an intermediate with an alternate leader segment serves as a cytoplasmic precursor in the absence of the y leader segment. Additional leader segments in cytoplasmic mRNA encoded at 75, 77, and 85 m.u.

have been observed in cells infected with Ad2 (8) and Ad2⁺ND4 (35). If this is the case, then we would expect to observe a significant fraction of pulse-labeled fiber mRNA possessing these leader segments.

Poly(A)-containing cytoplasmic RNA was isolated from cells infected with Ad2 or Ad2⁺ND5, pulse-labeled for 2 h with ^{32}P or pulse-labeled for 2 h and chased for 8 h in phosphate-containing medium. RNA samples were hybridized to immobilized SV40 DNA or Ad2 DNA specific for fiber mRNA. Selected RNAs were denatured with glyoxal and analyzed by agarose gel electrophoresis. An autoradiogram of the gel is shown in Fig. 5. No RNA species from Ad2-infected cells were selected by SV40 DNA, as expected. Two major species (4,900 and 3,200 bases) isolated from Ad2⁺ND5-infected cells were selected by SV40 DNA. These species were also selected by Ad2 L4-specific DNA and probably represent 100K and pVIII mRNAs, which are 3' coterminal at the SV40 poly(A) site (J. Ramsey, unpublished data).

A single discrete species was selected by fiber-specific DNA (83.4 to 89.7 m.u.) from RNA isolated from pulse-labeled cells infected with Ad2⁺ND5. This is in contrast to the heterogeneous fiber-specific species isolated from Ad2-infected cells. In addition, the Ad2⁺ND5 fiber mRNA (pulse-labeled or pulse-labeled and chased) comigrated with the most rapidly migrating form of fiber mRNA isolated from Ad2-infected cells, suggesting that it does not possess an additional leader segment.

To confirm this, pulse-labeled Ad2⁺ND5 fiber mRNA selected by hybridization to Ad2 83.4-to-89.7-m.u. DNA was hybridized a second time to

FIG. 5. Size analysis of fiber mRNA isolated from Ad2⁺ND5-infected cells. Cytoplasmic poly(A)-containing RNA was isolated from HeLa cells infected with Ad2 or Ad2⁺ND5 which had been pulse-labeled for 2 h with ^{32}P or pulse-labeled and chased for 8 h. SV40-specific RNAs and fiber mRNA were isolated by hybridization to immobilized SV40 DNA and Ad2 83.4-to-89.7-m.u. DNA, respectively. Selected RNAs were denatured with glyoxal and analyzed on an agarose gel.

FIG. 6. Hybridization of Ad2⁺ND5 fiber mRNA to various regions of the Ad2 genome. ³²P-pulse-labeled fiber mRNA from Ad2⁺ND5- or Ad2-infected cells was isolated by preparative hybridization to immobilized Ad2 83.4-to-89.7-m.u. DNA. Selected RNA was then hybridized a second time to DNA from various regions of the Ad2 genome as indicated, and RNA selected by this second hybridization was analyzed on an agarose gel.

DNA spanning the Ad2 genome from 21.5 to 83.4 m.u., and selected RNAs were analyzed by agarose gel electrophoresis (Fig. 6). Hybridization was only observed with DNA from 21.5 to 28.5 m.u., the region which encodes the third common leader segment. The slower-migrating fraction of pulse-labeled Ad2 fiber mRNA species hybridized to 75.9-to-83.4-m.u. DNA, as observed previously. Thus, we can find no evidence in cells infected with Ad2⁺ND5 of a major pulse-labeled fiber mRNA that contains a leader segment in addition to the common three-segment leader.

DISCUSSION

The presence of multiple fiber mRNAs in the cytoplasm of Ad2-infected HeLa cells was first described by Chow and Broker (8). The existence of heterogeneity in the spliced 5'-leader segments is necessarily due to the utilization of alternate pathways in the splicing of the primary transcript of the major late transcription unit. Chow and Broker (8) and Kilpatrick et al. (20) suggested that cytoplasmic adenoviral mRNAs possessing extra leader segments may represent intermediates in the formation of mature mRNAs. Because these species are found on polysomes and therefore are clearly of cytoplasmic origin, final processing of these species may occur in the cytoplasm.

Our results indicate that although fiber mRNAs possessing extra leader segments are readily detected in the cytoplasm, there is no evidence to suggest that they serve as precursors to mature fiber mRNA. This conclusion is

supported by kinetic and structural data which show that the size change in the cytoplasmic fiber mRNAs is due exclusively to metabolism of the 3'-poly(A) tail and that species containing extra leader segments are present at approximately the same relative frequencies after a 2-h pulse or after a 2-h pulse and an 8-h chase. The roughly equivalent cytoplasmic stabilities of the multiple fiber mRNAs argue against a precursor-product relationship, and the observation that the absence of most of these more complex fiber mRNAs in HeLa cells infected with Ad2⁺ND5 is not grossly deleterious to viral replication supports this conclusion.

Other investigators have examined the possibility of cytoplasmic splicing in a number of systems. Nevins (30) has shown that all of the late Ad2 mRNAs of the 38-to-51-m.u. mRNA family are formed in the nucleus and that the larger members of the family are not converted to the smaller members by splicing in the cytoplasm. Our results (Fig. 1) also support this conclusion; in fact, we have not detected any other potential cytoplasmic splicing events among the Ad2 late mRNAs. Piper et al. (33) have investigated the synthesis and processing of the three polyoma late mRNAs and have likewise concluded that each of these mRNAs is formed in the nucleus. An early report that the SV40 late 19S mRNA is processed in the cytoplasm to form the 16S species (4) has not subsequently been substantiated. Melton et al. (29) have examined the synthesis of yeast tRNA^{tyr} in *Xenopus laevis* oocytes and concluded that the tRNA precursor is spliced in the nucleus.

The role of alternate forms of viral mRNAs (if any) in viral replication is still of interest. Such species may in fact be nuclear intermediates in RNA processing which are transported to the cytoplasm before completion. The fact that these species represent only a small subset of nuclear processing intermediates would suggest that this transport is not random; i.e., these intermediates have a structure that makes them competent for transport which other nuclear species do not possess. In fact, we have observed a similar selective premature transport of precursors to cellular mRNA in Ad2-infected cells (C. Lawrence, unpublished data).

Species with extra segments could also be true processing endpoints which result from having alternate processing pathways for a single transcript. If the various cytoplasmic forms of a mRNA are true endpoints they might be expected to be functionally distinct. The additional y leader on fiber mRNA has no AUG codon to initiate protein synthesis (12) and is translated in vitro with the same efficiency as mature fiber mRNA, suggesting that there is no obvious functional significance to the presence of the y

leader. On the other hand, the "i" leader, which is present on a large fraction of late region 1 mRNA synthesized early in infection, probably encodes a 14K polypeptide (1). Determination of the sequences of other mRNA variants will at least indicate their potential for encoding different polypeptides.

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