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On the localization and transport of specific adenoviral mRNA-sequences in the late infected HeLa cell

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ABSTRACT

In the nucleus of HeLa cells late after infection with adenovirus type 2 mRNA-sequences which are processed via RNA splicing are attached to the nuclear matrix (Mariman et al., 1982). Although the mRNA, which codes for polypeptide IX, is not formed via splicing, about 70% of the non-poly-adenylated pre-mRNA and the polyadenylated pIX mRNA are bound to the matrix structure, indicating that polyadenylation is performed while the RNA is associated with the matrix. Binding to the nuclear matrix seems to be a common property of all mRNA-sequences in the nucleus. At the late stage of infection most of the newly synthesized mRNAs which appear in the cytoplasm are viral specific (Beltz & Flint, 1979). Kinetic analysis of the newly synthesized poly(A)-containing mRNA on sucrose gradients reveals that 7-12 S messengers appear more rapidly in the cytoplasm than messengers larger than 13 S. More specifically, the nuclear exit time of the pIX-mRNA, which is the major 9 S adenoviral messenger late after infection, was determined to be about 4 min, while messengers transcribed from the late region 3 need more than 16 min to arrive in the cytoplasm. In the cytoplasm about 70% of the mRNA is bound to the cytoskeletal framework, while 30% remains as free mRNP. Analysis of the mRNA in both fractions reveals that L3-, E1B- and pIX-specific polyadenylated mRNA preferably exist as cytoskeleton-bound mRNA. However, significant differences occur in the partition of specific messengers over free and cytoskeletal RNA fractions.

INTRODUCTION

It has been reported that hnRNA is quantitatively associated with a higher ordered proteinaceous structure, generally referred to as the nuclear matrix (1-4). At the late stage of infection of HeLa cells with adenovirus type 2 the viral specific hnRNA is also quantitatively attached to the nuclear matrix (4). It has been suggested that the nuclear matrix may function in localizing and structurally organizing hnRNA during its processing, since both mRNA-precursors and spliced products can be detected in the matrix associated RNA (5). After completion of its processing the matured messenger RNA is transported to the cytoplasm where it is translated.
mRNA-sequences in the nucleus as well as in the cytoplasm are complexed with several proteins. However, a different set of proteins seems to be associated

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with mRNA-sequences in the nucleus as compared to cytoplasmic mRNA, indicating that during nucleo-cytoplasmic transport one set of proteins is exchanged for another (6). Finally, in the cytoplasm mRNA can exist either as free mRNP or be bound to the cytoskeleton, a cytoplasmic framework composed of structural proteins, which can be isolated by treatment of cells with a non-ionic detergent (7, 8).

In this study we pursued specific adenoviral mRNA-sequences from transcription to their destination in the cytoplasm. Although all adenoviral mRNA-sequences roughly seem to follow the same pathway through the infected HeLa cell, significant differences between specific mRNAs occur in the rate of their nucleo-cytoplasmic transport and their localization in the cytoplasm.

MATERIALS AND METHODS

Infection and labeling of cells

HeLa S3 cells were infected with adenovirus type 2 (2000 particles per cell) at a density of $5x10^6$ cells/ml in Eagles minimal essential medium containing 1 mM arginine (MEM). After adsorption of the virus to the cells during 1 h at 37 ^oC, the cells were diluted to $0.3x10^6$ cells/ml with MEM containing 5% newborn calf serum. Cells were harvested at 18 h after infection. To obtain a time course of labeled mRNA 18 h after infection, cells were concentrated to $20x10^6$ cells/ml and incubated for the indicated periods of time with 50 µCi/ml (5,6-^3H) uridine (45 Ci/mmol, Radiochemical Centre Amersham, England), 25 µCi/ml (5'-^3H) guanosine (20 Ci/mmol, Amersham) and 25 µCi/ml (5-^3H) cytidine (31 Ci/mmol, Amersham). To obtain steady state labeled mRNA the cells were labeled from 16 to 18 h after infection with 5 µCi/ ml (5,6-^3H) uridine at a density of $4x10^6$ cells/ml.

Purification and oligo(dT)-cellulose chromatography of cytoplasmic and nuclear RNA

The incorporation of labeled precursors in the RNA was terminated by rapidly mixing the cells with an equal volume of crushed, frozen NKM solution (0.13 M NaCl, 0.05 M KCl, 1.5 mM $MgCl_2$). After centrifugation for 5 min at 800g the cells were washed once with NKM, pelleted and resuspended in RSB (10 mM NaCl, 10 mM Tris, pH 7.4, 1.5 mM $MgCl_2$). After the addition of 0.1 vol of a sodium deoxycholate-Nonidet P40 mixture (5% each) the cells were disrupted by intensive vortexing. The nuclei were pelleted and from the supernatant cytoplasmic RNA was isolated by phenol/chloroform extraction (9). The nuclei were washed once in RSB and resuspended in the same buffer. Nuclear RNA was isolated by phenol extraction at 55^oC as described by Long et al.

(10). To select the poly(A)-containing RNA oligo(dT)-cellulose chromatography was performed as described by Aviv and Leder (11) using the T2 grade of Collaborative Research Inc.

Fractionation of cytoplasmic RNA into free and cytoskeleton-bound mRNA

Cytoskeletons from HeLa cells were prepared essentially as described by van Venrooij et al. (12). Cells were harvested by centrifugation and washed once with isotonic NKM solution and once with hypertonic buffer (0.3 M sucrose 10 mM KCl, 1.5 mM Mg-acetate, 10 mM Tris-acetate, pH 7.4). The cells were resuspended in hypertonic buffer ($40x10^6$ cells/ml) and 1 vol. of 1% Triton X-100 (BDH Chem. Ltd., Poole, Dorset) in hypertonic buffer was added. The suspension was gently swirled in ice for 30 sec and centrifuged (5 min, 800g) to sediment the cytoskeletons. The supernatant containing the free mRNA was pipetted off and the pellet was washed with hypertonic buffer containing 0.5% Triton X-100. After centrifugation the pellet was resuspended in RSB. To solubilize the cytoskeletal fraction 0.1 vol. of a sodium deoxycholate-Nonidet P40 mixture (5% each) was added to the suspended cytoskeletons and the suspension was intensively vortexed. The nuclei were removed from the cytoskeletal fraction by centrifugation at 800g for 5 min. Immediately after cell fractionation the various fractions were made 1% in SDS and deproteinized by phenol/chloroform extraction (9). The RNA was then passed over oligo(dT)-cellulose (T2-grade, Collaborative Research Inc.) to select the poly(A)-containing mRNA (11).

Preparation of nuclear matrix RNA

Nuclear matrices of HeLa cells 18 h after infection were isolated as previously described (4). Nuclei were isolated from infected HeLa cells as described above. The nuclei were resuspended in RSB containing 0.1 M NaCl and treated with DNAse I for 30 min at 10^oC (500 µg/ml). After DNAse I digestion the nuclei were pelleted through a 30% sucrose layer by centrifugation at low speed. The pelleted material was extracted once with 0.4 M $(NH_4)_2SO_4$. After repelleting the nuclear matrices were washed with RSB, suspended in RSB buffer containing 1% SDS and incubated at 100^oC for 3 min. The boiled mixture was adjusted to 0.2 M NaCl and extracted with phenol/chloroform (1:1). After two additional extractions with chloroform the RNA was precipitated with two volumes of ethanol.

Adenoviral DNA isolation and restriction endonuclease digestions

Viral DNA was isolated from the purified virions as described by Petterson and Sambrook (13). EcoRI and HindIII (Boehringer, Mannheim) restriction digestions were performed at 37^oC for 4 h using 1 unit of enzyme per µg DNA.

Standard conditions were 50 mM Tris, pH 7.4, 7 mM $MgCl_2$, 50 mM NaCl, 3 mM DTT. SstI (Bethesda Research Laboratories Inc.) digestions were performed at 37°C for 12 h (1 unit/µg DNA) in Tris buffer containing 10 mM Tris, pH 7.4, 7 mM $MgCl_2$, 10 mM KCl, 20 mM NaCl, 3 mM DTT. In case of SmaI (Boehringer, Mannheim) the DNA was digested at 25°C for 12 h (1 unit/µg DNA) in a buffer containing 15 mM Tris, pH 8.5, 6 mM $MgCl_2$, 15 mM KCl, 3 mM DTT. Restriction fragments were separated by agarose gel electrophoresis; 0.8% agarose gels were run for 20 h at 80 V in buffer TNE (40 mM Tris, pH 8.0, 8 mM NaAc, 2 mM EDTA). The DNA was recovered from agarose gel slices by the method of electrophoretic elution.

RNA-DNA hybridization

The DNA restriction fragments were immobilized to nitrocellulose filters as described by Gillespie and Gillespie (14). RNA was dissolved in 200 µl 3x SSC (SSC, 0.15 M sodium chloride-0.015 M sodium citrate), 1 mM EDTA, 0.1% SDS, 50% formamide. To this solution a filter containing 2 µg equivalent of DNA of a particular restriction fragment was added. The RNA was denatured by incubating the mixture at 65°C for 5 min. Hybridization was then carried out at 38°C for an appropriate period of time. After hybridization the filters were washed several times in 2x SSC and subsequently with 0.1 N NaOH at 45°C for 10 min. The solution containing the eluted RNA was then neutralized with HCl and diluted with one volume of water. The radioactivity in the hybridized RNA was determined by means of liquid scintillation counting using pico-fluor™15 (Packard Inst.) as a scintillant.

RESULTS

Association of mRNA-sequences with the nuclear matrix

It has been shown that the nucleus of HeLa cells, as well as of other cell types, contains a higher ordered proteinaceous structure, usually referred to as the nuclear matrix, to which the hnRNA is quantitatively attached (1-4). Recently it was shown that the process of RNA splicing is performed while the RNA is attached to the matrix structure (5). With this finding the possibility arose that only mRNA-sequences which have to be spliced are bound, for example via intron sequences, to the matrix structure. Release of processed mRNA would then occur simultaneous with the final splicing step. Unspliced mRNAs, in that case, would not be found associated with the matrix. The messenger which codes for polypeptide IX (15), a structural component of the virion, is transcribed from the viral r-strand between coordinates 9.8 and 11.2 (16; the viral genome is divided into 100 map units). Recently it was shown that the nucleotide sequences of this mRNA is colinear with the DNA (17) and until

now this messenger is the only known adenoviral mRNA which is unspliced.

To test our hypothesis HeLa cells 18 h after infection with Ad2 were labeled with ^{3}H-nucleosides for 30 min. After labeling the nuclei were isolated as indicated in Materials and Methods and divided in two. From one part nuclear RNA was isolated by means of the hot phenol extraction procedure (see Materials and Methods). From the other part of the nuclei nuclear matrices were prepared. The nuclear matrix RNA was then isolated as described in the Materials and Methods section. Both nuclear and nuclear matrix RNA were fractionated in polyadenylated (6% of the labeled RNA) and non-polyadenylated RNA (94% of the labeled RNA) via oligo(dT)-cellulose chromatography. This demonstrates that not only polyA(+) but also polyA(-) hnRNA is bound to the nuclear matrix. The amount of RNA containing nucleotide sequences specific for L3, E1B or pIX in each fraction was then quantitated by the method of filter hybridization as described in the Materials and Methods section. The nucleotide sequences of the pIX-specific RNA reside in the region E1B (18; 4.6-11.2 m.u.), which is active in transcription at the early stage of infection. However, transcription of this region seems to continue in the late phase of infection. Two early messengers are transcribed from the region E1B which both contain the nucleotide sequences that make up the mRNA for pIX (18; Fig. 1A). Nuclear and nuclear matrix RNA were hybridized to a HindIII/SmaI restriction fragment of the Ad2 DNA (7.9-10.8 m.u.; Fig. 1A), which binds the E1B-specific RNA as well as the pIX-specific RNA-sequences. The RNA was also hybridized to a SstI/HindIII fragment (5.0-7.9 m.u.; Fig. 1A), which only detects the E1B-specific RNA. The percentage of hybridization due to the pIX RNA was calculated by substracting the percentage found with the Sst/Hind fragment from the percentage that was found when the Hind/Sma fragment was used. To detect the L3-specific RNA a Hind/EcoRI restriction fragment (19; 50.1-58.5 m.u.; Fig. 1B) was used. The results, listed in table I, show that 70% or more of the nuclear RNA, which contains L3-, E1B- or pIX-specific sequences is attached to the nuclear matrix. There is no significant difference in the binding to the nuclear matrix between RNA which contains sequences for the unspliced pIX mRNA and the RNA sequences which form the L3 or E1B messengers via splicing. Therefore, binding to the nuclear matrix seems to be a common property of mRNA sequences, that have to be transported to the cytoplasm and is not limited to the messengers which are generated via splicing.

Sucrose gradient analysis of newly accumulated mRNA in the cytoplasm

At the late stage of infection of HeLa cells with Ad2 most of the newly synthesized mRNAs, which appear in the cytoplasm are viral specific (20).

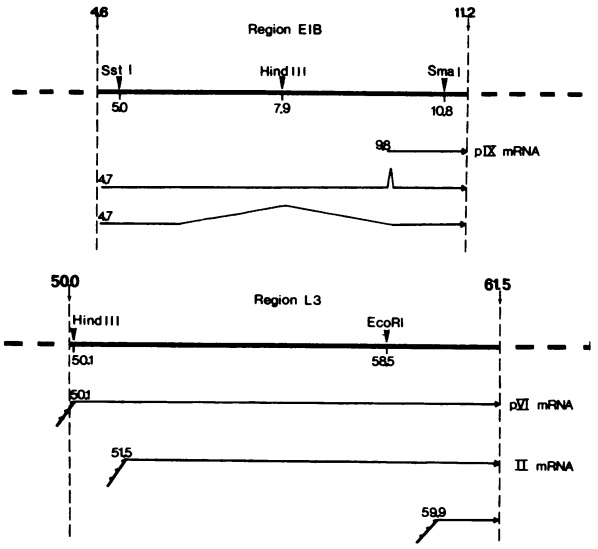

Fig. 1. A. Map coordinates of the early region 1B. The mRNAs which are transcribed from the region E1B are represented by arrows, arrowheads point at the 3' end of the mRNAs. Caret symbols indicate the intervening sequences which are absent from the spliced messengers. The cleavage sites of the endonuclease restriction enzymes which have been used to obtain Ad2 DNA fragments, are as indicated. B. Map coordinates of the late region 3. The mRNAs which are transcribed from the region L3 are represented by arrows, arrowheads point at the 3' end of the mRNAs. Jointed stalks represent the spliced tripartite leader sequences. The cleavage sites of the endonuclease restriction enzymes which have been used to obtain Ad2 DNA fragments, are as indicated.

Figure 2 shows the sedimentation profile of steady state mRNA late after infection as determined by sucrose gradient centrifugation. Three size classes can be distinguished: 18-24 S (class I), 14-17 S (class II) and 7-12 S (class III). The first type of experiment we undertook was to see if there were appreciable differences in the time of appearance in the cytoplasm between mRNAs of different size classes. Therefore, HeLa cells 18 h after infection were labeled with ^{3}H-nucleosides (see Materials and Methods) for 5, 15, 30 or 45 min, respectively. The poly(A)-containing cytoplasmic RNA was isolated, denatured and layered on top of a 16-43.5% (w/v) isokinetic sucrose gradient. After centrifugation the gradients were fractionated and the radioactivity in each fraction was determined. As can be seen from the gradient profiles in Figure 3 class III mRNA appears quite rapidly in the cytoplasm (Fig. 3A). After labeling for 30 min or longer the gradient profile (Fig. 3C and 3D) already closely resembles that for steady state mRNA (Fig. 2)

Table I. Determination of the percentages of L3-, E1B- and pIX-specific nuclear RNA sequences which are bound to the nuclear matrix.

		Hybridization of nuclear RNA (%)	Hybridization of matrix RNA (%)	Nuclear RNA bound to the matrix (%)	Poly A(+)/ poly A(-)
L3-specific	pA^+	7.1	6.1	86	0.8
	pA^-	9.8	7.6	78	
E1B-specific	pA^+	7.4	5.3	72	0.9
	pA^-	6.7	5.7	85	
pIX-specific	pA^+	1.0	0.7	70	0.2
	pA^-	3.8	3.2	84	

Ad2 infected HeLa cells 18 h after infection were labeled with ^{3}H-nucleosides for 30 min. After labeling nuclear and nuclear matrix RNA were prepared and fractionated in polyA(+) and polyA(-) RNA by oligo(dT)-cellulose chromatography (Materials and Methods). In each fraction of RNA the amounts of L3-, E1B- and pIX specific sequences were determined by filter hybridization as described in the Materials and Methods section. The restriction fragments of Ad2 DNA which were used, are as indicated in the text. The percentages of hybridization were corrected for non-specific adsorption by incubation of RNA with a blank filter under the same conditions. The third column shows the percentages of specific RNA sequences which are bound to the nuclear matrix. These data were calculated from the amounts of certain specific RNA sequences in nuclear and nuclear matrix RNA as shown in the first and second column. The fourth column shows the ratio between the amounts of certain specific RNA sequences in polyA(+) and polyA(-) nuclear matrix RNA. Each percentage is the mean value of the results obtained in 5 separate hybridizations. The deviation of the values in the first and second column is within 1/10 of the indicated values.

while at the intermediate labeling time of 15 min the size distribution also was found to be intermediate (Fig. 3B). The ratios between the amounts of newly synthesized class I and class III mRNAs and between class II and class III mRNAs rise between 5 and 45 min labeling illustrating the fact that there is a significant difference between the time of appearance in the cytoplasm of 7-12 S poly(A)-containing messengers on one hand and messengers larger than 13 S on the other.

Nuclear exit times of different adenoviral messengers

The gradient profiles of Figure 3 showed that 7-12 S messengers appear more rapidly in the cytoplasm than larger mRNAs. We were interested to see if this result could be verified for specific mRNAs by determining the nuclear exit

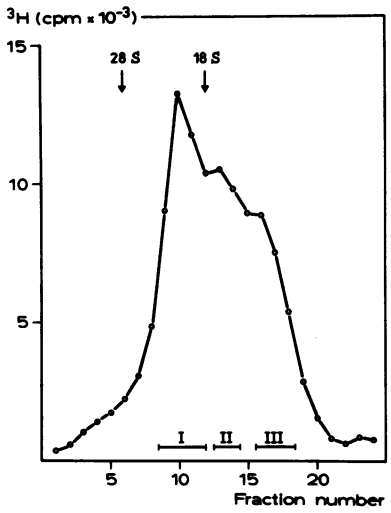

Fig. 2. Sucrose gradient profile of steady state labeled poly(A)-containing cytoplasmic RNA. HeLa cells were infected with Ad2 and from 16 to 18 h after infection the cells were labeled with 5 µCi/ml (5,6-^{3}H) uridine. The cytoplasmic RNA was isolated as described in the Materials and Methods section. To isolate the poly(A)-containing fraction of the cytoplasmic RNA, oligo(dT)-cellulose chromatography was performed as described by Aviv and Leder (11). The RNA was dissolved in a buffer of low ionic strength containing 10 mM Tris, pH 7.4, 2 mM EDTA, 0.2% SDS, heated to 100°C for a few minutes, and after rapid cooling the solution was layered on top of a 16-43.5% (w/v) isokinetic sucrose gradient containing the same buffer. Centrifugation was for about 16 h at 20°C in a SW41 rotor at 150,000g. The gradient was fractionated and the radioactivity counted with pico-fluor™15 (Packard Inst.). The horizontal bars indicate the different size classes of RNA which can be distinguished in the gradient profile.

time of the messenger for pIX, which is the major 9 S adenoviral messenger at the late stage of infection (15), and that of the messengers which are transcribed from the late region 3 (Fig. 1B) and which sediment at a higher density than 18 S (15). HeLa cells 18 h after infection were labeled for 4, 8, 12, 16 min and 5, 15, 30, 45 min, respectively. Poly(A)-containing cytoplasmic RNA was isolated and hybridized to appropriate restriction fragments of the Ad2 DNA, immobilized to nitrocellulose filters, as described in the first section of this paragraph (Fig. 1; Materials and Methods). The same quantities of RNA were, under the same conditions, also hybridized to filters containing 2 µg of total Ad2 DNA. The percentages of RNA hybridized to restriction fragments were then divided by the percentages of RNA hybridized to total Ad2 DNA.

The results are depicted in Figure 4 and in agreement with our expectation they show that the first pIX-specific sequences appear in the cytoplasm already after 4 min labeling, while labeled L3 messengers begin to accumulate after 16 min (Fig. 4).

Partition of messengers over free and cytoskeleton-bound mRNA fractions

Morphological studies including immunofluorescence and electron microscopy have revealed the existence of a cytoskeletal framework composed

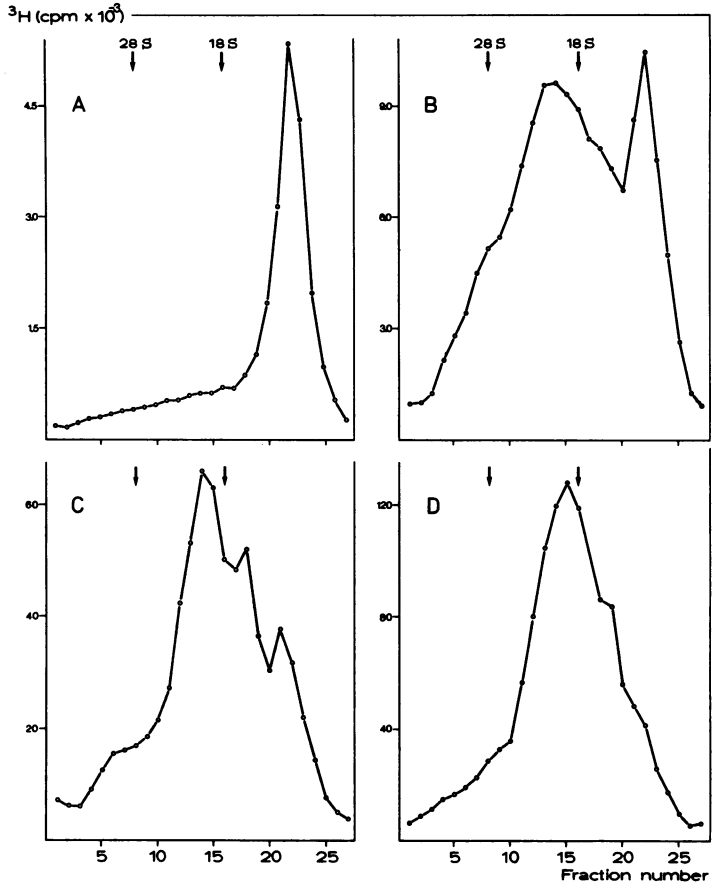

Fig. 3. Sucrose gradient analysis of newly accumulated poly(A)-containing mRNA in the cytoplasm. HeLa cells were infected with Ad2. After labeling the cells with ^{3}H-nucleosides (see Materials and Methods) for a period of time as indicated in the text, 18 h after infection, the poly(A)-containing cytoplasmic RNA was isolated and analysed on an isokinetic sucrose gradient as described in the legend of Figure 2. Labeling time: 5 min (3A); 15 min (3B); 30 min (3C); 45 min (3D).

of structural proteins in the cytoplasm of eukaryotic cells, which can be isolated by gently extracting the cells with a non-ionic detergent such as TX-100 (7, 8). Some workers have found that cytoplasmic mRNA is in part bound to the cytoskeleton but that it can exist also as free mRNP. Further results have indicated some specificity in the distribution of mRNA-sequences over the free and cytoskeleton-bound mRNA fractions (12, 21, 22).

To determine if adenoviral mRNA preferably exists as free mRNP or as

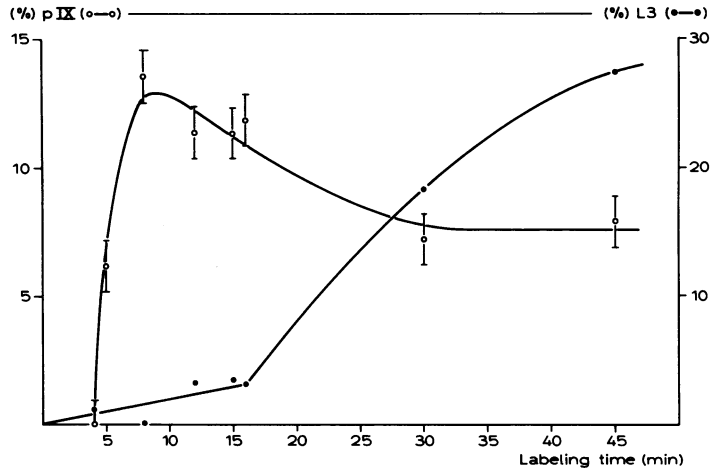

Fig. 4. Determination of nuclear exit times by filter hybridization. Ad2 infected HeLa cells 18 h after infection were labeled with ^{3}H-nucleosides for 4, 8, 12, 16 min and 5, 15, 30, 45 min respectively (Materials and Methods). Poly(A)-containing cytoplasmic RNA was prepared, dissolved in 200 µl 3x SSC, 1 mM EDTA, 0.1% SDS, 50% formamide and hybridized to DNA fragments immobilized to nitrocellulose filters. After hybridization the percentage of RNA which was hybridized to the DNA was determined as described in the Materials and Methods section. To detect L3-specific sequences the RNA was hybridized for 70 h to filters containing 2 µg equivalent of the HindIII/EcoRI fragment indicated in Figure 1B. The percentages were corrected for non-specific adsorption by incubation of a blank filter with RNA under the same conditions. To detect pIX-specific sequences the RNA was hybridized for 150 h to 2 µg equivalent of an SstI/HindIII as well as a HindIII/SmaI fragment (Fig. 1A). The percentages of hybridization due to pIX-specific sequences were calculated by substracting the percentages found with the first fragment from those which were found with the second fragment. Using identical conditions the same quantity of RNA was hybridized to filters containing 2 µg Ad2 DNA. The percentage of the RNA hybridized to a DNA frgment was divided by the percentage of the RNA which hybridized to total Ad2 DNA under these conditions. Each point in the curves is the mean value of 5 separate hybridizations.

cytoskeleton-bound mRNA, HeLa cells were labeled from 16 to 18 h after infection to obtain steady state labeled mRNA (Materials and Methods). After labeling poly(A)-containing mRNA was prepared from both the free and cytoskeletal RNA fraction as described in the Materials and Methods section. In accordance with earlier results (12) it was found that only 30% of the steady state labeled mRNA exists as free mRNP while as much as 70% is bound to the cytoskeleton. The sucrose gradient profiles as depicted in Figure 5 show that there is only a slight difference in the size distribution and composition between free and cytoskeletal mRNA. By the method of filter hybridization as described in the first section of this paragraph we

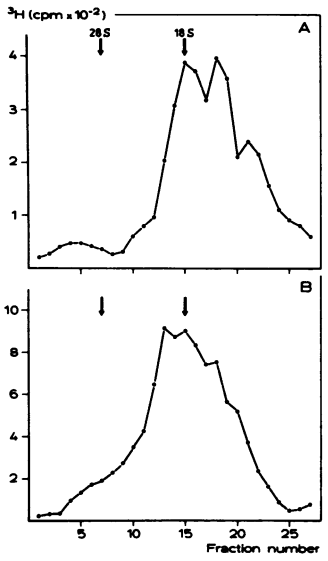

Fig. 5. Sucrose gradient analysis of steady state labeled polyA(+) mRNA isolated from the free and cytoskeletal RNA fractions. HeLa cells were infected with Ad2. After labeling the cells with (5,6-^{3}H) uridine (5 µCi/ml) from 16 to 18 h after infection, the free and cytoskeleton RNA fractions were isolated as described in the Materials and Methods section. Oligo(dT)-cellulose chromatography was performed (11) to isolate the poly(A)-containing RNA. The RNA was then dissolved in a buffer of low ionic strength containing 10 mM Tris, pH 7.4, 2 mM EDTA, 0.2% SDS, heated to 100°C for a few minutes, and after rapid cooling the solution was layered on top of a 16-43.5% (w/v) isokinetic sucrose gradient containing the same buffer. Centrifugation was for about 16 h at 20°C in a SW41 rotor at 150,000g. A. Free polyA(+) mRNA. B. Cytoskeleton-bound polyA(+) mRNA.

quantitated the amounts of L3-, E1B- or pIX-specific mRNA-sequences in both the free and the cytoskeletal mRNA fraction. The results (Table II) demonstrate that each of the individual mRNAs preferably is bound to the cytoskeletal framework. However, significant differences can be detected in the partition of specific messengers over both cytoplasmic fractions. This possibly reflects the affinity of individual messengers for the cytoskeletal framework but, as was suggested earlier (12), can also point to a regulation mechanism of the translation of specific (adenoviral) mRNAs.

DISCUSSION

The mRNAs of eukaryotic cells are usually derived from larger precursor molecules. These primary products of transcription are modified by a number of processing steps, i.e. capping, polyadenylation, methylation and splicing. After processing, which is performed in the cell nucleus, the newly formed mRNA is transported out to the cytoplasm. At some time during mRNA transport, the proteins which are associated with the mRNA in the nucleus are replaced by a cytoplasm-specific set of mRNA-associated proteins (6). In the cytoplasm messengers can occur either as free mRNP or be bound to the cytoskeleton, a cytoplasmic framework (12, 21, 22). In the present study we pursued some adenoviral mRNA-sequences from transcription to their destination in the cytoplasm. Our main results can be summarized as follows:

Table II. Partition of L3-, E1B- and pIX-specific poly(A)-containing mRNA over free and cytoskeletal RNA fractions.

	free RNA fraction	cytoskeletal RNA fraction
L3-specific mRNA	11.5%	88.5%
E1B-specific mRNA	2.2%	97.8%
pIX-specific mRNA	28.5%	71.5%

Ad2 infected HeLa cells were labeled from 16 to 18 h after infection with ^{3}H-nucleosides. After labeling the polyA(+) mRNA was isolated from both the free and cytoskeleton-bound RNA fractions (Materials and Methods). In each fraction of cytoplasmic mRNA the amounts of L3-, E1B- and pIX-specific mRNA were determined by filter hybridization as described in the Materials and Methods section. The restriction fragments of the Ad2 DNA, which were used, are indicated in the first section of this paragraph. The percentages of hybridization were corrected for non-specific adsorption by incubation of RNA with a blank filter under the same conditions. From these data the partition of specific mRNA sequences over the free and cytoskeletal RNA fractions were calculated. Each percentage is the mean value of the results of 5 separate hybridizations. The absolute deviation in the indicated percentages is less than 2.5%. Recently we have found the same results using 1 h labeled RNA and cloned DNA fragments.

1. In the nucleus pre-mRNA sequences, polyadenylated or non-polyadenylated, are associated with the nuclear matrix regardless of the fact that their processing pathway includes the process of RNA splicing or not.
2. Generally, small-sized messengers ($\pm$ 9 S) appear more rapidly in the cytoplasm than larger mRNAs. When specific mRNAs are compared, the nuclear exit time differs significantly.
3. In the cytoplasm messengers are preferably bound to the cytoskeletal framework although significant differences occur in the steady state distribution of specific mRNAs over free and cytoskeleton-bound RNA fractions.

HnRNA is quantitatively attached to a nuclear matrix (1-4). Adenovirus region L4 RNAs are attached to the nuclear matrix both as precursors and as products of splicing, indicating that splicing is performed while the RNA is associated with the matrix structure (5). Accordingly, using the method of filter hybridization for a more quantitative approach, we have found that at least 70% of the L3- and E1B-specific nuclear RNAs, which both are processed via RNA splicing, are attached to the nuclear matrix. Similarly it was shown that the polyA(-) nuclear matrix RNA contains at least 70% of the non-

polyadenylated pIX-specific sequences present in the nucleus. From this we concluded that pre-mRNA for the pIX messenger is attached to the nuclear matrix, although the mRNA is not formed via splicing. At least 70% of the nuclear poly(A)-containing pIX RNA is also attached to the nuclear matrix, indicating that not only splicing but also polyadenylation is performed while the RNA is bound to the matrix structure. From Table I it can be seen that polyA(+) and polyA(-) nuclear matrix RNA roughly contain the same amount of L3- or E1B-specific sequences. However, the polyA(+) fraction contains about 4 times less pIX-specific sequences than the polyA(-) fraction. This suggests that polyadenylation is the last step in the processing of pIX RNA and indicates that the mature mRNA, after polyadenylation, is immediately transported out to the cytoplasm. The measurement of the nuclear exit time of these specific sequences confirms this suggestion. As seems to be the case for most adenoviral mRNAs (23) processing and transport of mRNA-sequences of the late region 3 last more than 16 min. However, the processing and transport of pIX mRNA is performed in only about 4 min (Fig. 4). The relatively rapid appearance of pIX mRNA in the cytoplasm could be explained by the fact that the processing pathway does not include RNA splicing, which could be a time-consuming processing step. An alternative explanation would be that the rapid appearance of pIX mRNA could be the result of an enhanced rate of nucleo-cytoplasmic transportation. Our findings do not support the existence of different transport mechanisms for pIX and L3 messengers. However, rapid transport could simply be the result of the smaller size of the pIX messenger. The gradient profiles of Figure 3 show that small-sized messengers ($\pm$ 9 S) are the first to appear in the cytoplasm. This seems to be a more general feature since other workers have obtained similar results using uninfected HeLa cells and cultured L-cells (24, 25). They reported that 9 S mRNA accumulated within 10 min in the small polyribosomes of these cells. In addition it was reported that the β-globin mRNA, a 10 S mRNA which is formed via RNA splicing, reached the cytoplasm within 5 min after termination of transcription (26, 27). Studies on the accumulation of E1A and E1B mRNAs have been performed by Wilson and Darnell (28).

In the cytoplasm about 70% of the adenoviral poly(A)-containing mRNA is bound to the cytoskeletal framework. Binding to the cytoskeleton might be obligatory for the mRNA to be translated (12, 22). Sucrose gradient analysis of free and cytoskeletal polyA(+) mRNA late after infection reveals that both fractions of RNA sediment with roughly the same velocity (Fig. 5). However, translation experiments have indicated that among adenovirus-specific

messengers there may be species which exist preferably as free or as cytoskeletal mRNA (12). Using filter hybridization we found that L3-, E1B- and pIX-mRNA species are preferably bound to the cytoskeletal framework (Table II). However, while the E1B mRNA was almost quantitatively bound to the cytoskeletal framework, about 30% of the pIX mRNA was present as free mRNP. This demonstrates that among specific mRNA species there are differences in the binding to the cytoskeletal framework supporting the hypothesis that cytoskeleton-binding may be a regulatory step in the synthesis of (viral) proteins (12, 21, 22).

Summarizing, our results show that various adenoviral mRNA-sequences seem to follow a similar pathway through the infected HeLa cell, notwithstanding large differences in their nuclear processing, their exit time and their partition over free and cytoskeleton-bound mRNA fractions.

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