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Cell-free Synthesis of Adenovirus 2 Proteins Programmed by Fractionated Messenger RNA: A Comparison of Polypeptide Products and Messenger RNA Lengths

(sodium dodecyl sulfate–polyacrylamide gel electrophoresis/fingerprint analysis/formamide)

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ABSTRACT **Cytoplasmic RNA extracted from human tissue culture cells infected with adenovirus type 2 was used to program protein synthesis in a cell-free system derived from mammalian cells. Analysis of the protein product by polyacrylamide gel electrophoresis revealed ten adenovirus-specific polypeptides. Five of these were further identified by analysis of tryptic peptides. Translation of RNA fractionated by sedimentation through sucrose gradients containing formamide demonstrated seven size classes of RNA, each of which programmed the synthesis of only one or two virus-specific polypeptides. Six of the virus-specific polypeptides were translated from RNAs much larger than expected for the size of the polypeptide.**

Considerable progress has recently been made in developing cell-free systems capable of translating mammalian messenger RNAs (1). Such systems promise to be useful for studying the expression of viral genes in host cells, including those genes involved in transformation of cells by tumor viruses.

Adenovirus 2 (Ad2) (for review see ref. 2) is a human virus which is capable of transforming cells *in vitro*. The viral DNA has a molecular weight of 23×10^6, sufficient to encode 20 to 50 proteins. Adenovirus infection inhibits synthesis of host macromolecules, simplifying the identification of adenovirus-specific products. Approximately eighteen virus-specific proteins have been found in extracts of infected cells (2, 3). Six of these are major constituents of the virus particle and have been well characterized (2). Discrete classes of early and late viral mRNA have been demonstrated by fractionation according to size (4, 5) and homology with specific DNA fragments (6), but these have not been identified with their polypeptide products.

Cell-free synthesis of adenovirus proteins was first demonstrated using polysomes from virus-infected cells (7, 8). In this paper we show that the RNA fraction from Ad2-infected cells, when added to a mammalian cell-free system, results in synthesis of specific Ad2 proteins. We have used cell-free protein synthesis to identify the mRNA size class that directs the synthesis of each viral polypeptide.

METHODS

RNA Extraction. RNA was extracted from uninfected KB cells and from KB cells 30 hr after adenovirus infection (3) by phenol:chloroform extraction (9), modified as follows. The cells were collected by centrifugation at $1000 \times g$ for 10 min at 0°, and washed once with phosphate-buffered saline. The packed cells were resuspended in 4–10 volumes ice-cold Tris-saline (0.01 M Tris·HCl, pH 7.0, 0.15 M NaCl, 2 mM $MgCl_2$), and Nonidet P40 (Shell) was added to 0.5%. After 3–5 min the nuclei were removed by centrifugation at $1000 \times g$ for 5 min and the cytoplasmic extract was added to an equal volume of TSE buffer (0.01 M Tris·HCl, pH 7.6, 0.15 M NaCl, and 5 mM ethylenediaminetetraacetate) containing 1% sodium dodecyl sulfate. This mixture was then extracted three times with phenol:chloroform:isoamyl alcohol (24:24:1) saturated with TSE buffer. The RNA was precipitated twice with 2 volumes of ethanol at −20°, resuspended at a concentration of 200 A_{260} units/ml in H_2O, and stored at −20°. Globin RNA (9 S) was prepared as described by Lingrel (1).

The Mammalian Translation System. The translation system used was that of Schreier and Staehelin (10) with the following modifications.

Although mouse liver subunits and rat liver pH 5 enzyme were satisfactory, we used the corresponding components prepared from Ehrlich or Krebs II ascites cells. Postmitochondrial supernatant from these cells was prepared by the method of Mathews and Korner (11) or by the following variation. Cells were sedimented and resuspended in 2 volumes of 0.01 M KCl, 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 0.01 M Tris·HCl (pH 7.6), and 0.2 mM ethylenediaminetetraacetate. After 5 min at 0° the cells were disrupted in a glass Dounce homogenizer with 25 strokes of the tight-fitting plunger. Immediately one volume of 1.0 M sucrose, 0.02 M KCl, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 0.01 M Tris·HCl (pH 7.6) was added, and the extract was centrifuged. Either method was satisfactory for preparing ribosomal subunits, but only the second procedure was suitable for preparing pH 5 enzyme. Ribosomal subunits were prepared from polysomes as described (10) except that the incubation for polysome run-off was done directly with the postmitochondrial supernatant fraction (final A_{260} of 60 in the reaction mixture). The separated subunits were pooled (60 S to 40 S in an A_{260} ratio of 2.5:1), sedimented, resuspended at 100 A_{260} units/ml, and stored at −70° in 0.1-ml aliquots (without pH 5 enzyme). An S-100 fraction prepared from ascites postmitochondrial supernatant was used to prepare pH 5 enzyme (10).

Abbreviation: Ad2, adenovirus type 2.

Initiation factors from rabbit reticulocytes were prepared by the method of Schreier and Staehelin (10) except that the crude fraction eluted from ribosomes with 0.5 M KCl was first separated into fractions precipitating at 30–40% and 40–70% saturation with $(NH_4)_2SO_4$. The 30–40% $(NH_4)_2SO_4$ fraction was further purified by DEAE-cellulose chromatography; the material that eluted between 0.15 and 0.25 M KCl was used.

Assay for Protein Synthesis. Standard reaction mixtures of 50 μl contained 0.25 A_{260} units purified ribosomal subunits, 5 μl of pH 5 enzyme (containing 65 μg of protein and 4 μg of RNA), 12 μg of initiation factors precipitating at 30–40% $(NH_4)_2SO_4$ saturation and purified by DEAE-cellulose, and 50 μg of 40–70% $(NH_4)_2SO_4$ initiation factors. The other ingredients were 1.0 mM ATP; 0.4 mM GTP; 10 mM creatine phosphate; 20 μg/ml of creatine kinase; 30 mM Tris·HCl (pH 7.6); potassium, magnesium, and RNA at the concentrations given below; and 30 μM each of 19 amino acids minus leucine and with 15 μM [^{14}C]leucine at 0.1 Ci/mmole, or, for product analysis, minus methionine and with 2 μM [^{35}S]-methionine at greater than 100 Ci/mmole. Reaction mixtures were incubated for 1 hr at 35°.

RESULTS

Protein Synthesis Directed by Ad2 RNA. The incorporation of amino acids into protein in the mammalian cell-free system of Schreier and Staehelin was stimulated by addition of cytoplasmic RNA from KB cells infected with Ad2 (Fig. 1). Maximal synthesis was observed with 16–24 μg of RNA per 50-μl reaction mixture (only 3–5% of the total RNA was Ad2 mRNA). RNA enriched for poly(A)-containing molecules by chromatography on oligo(dT)-cellulose (12) also stimulated amino-acid incorporation and gave protein products indistinguishable from those synthesized on addition of unfractionated RNA. Most of the synthesis occurred during the first 60 min of incubation (Fig. 1*B*), but synthesis continued for 2 hr.

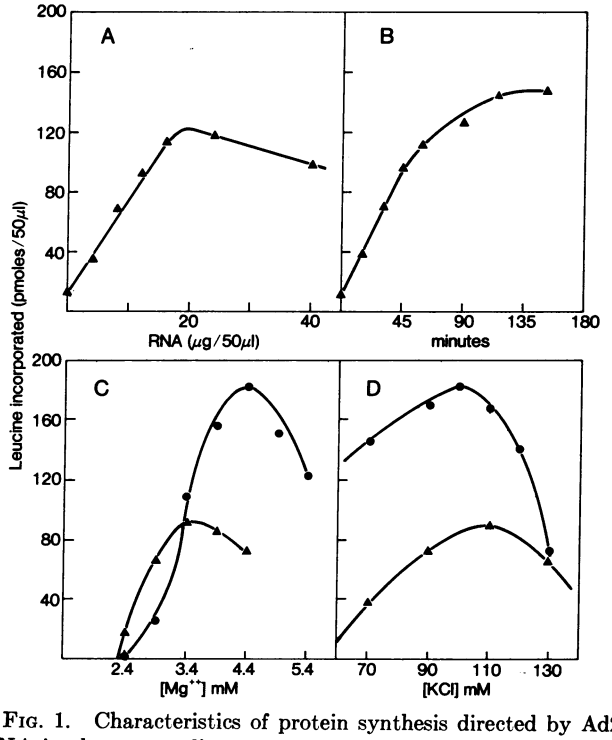

FIG. 1. Characteristics of protein synthesis directed by Ad2 RNA in the mammalian system. (*A*) Response to added cytoplasmic RNA from Ad2-infected cells at 3.4 mM Mg^{2+} and 110 mM K^+. (*B*) Time course of protein synthesis at 3.4 mM Mg^{2+}, 110 mM K^+, and 15 μg of Ad2 RNA/50μl. (*C*) Effect of magnesium concentration on protein synthesis at 110 mM K^+ for 12 μg of Ad2 RNA/50 μl (▲) and for 0.5 μg of rabbit globin mRNA/50 μl (●). (*D*) Effect of potassium concentration on protein synthesis for 12 μg of Ad2 RNA/50 μl (▲) at 3.4 mM Mg^{2+} and for 0.5 μg of rabbit globin mRNA/50 μl at 4.4 mM Mg^{2+} (●).

Product Analysis. Under our conditions a 50-μl reaction mixture containing mRNA incorporated 3.0×10^6 cpm from [^{35}S]methionine (15% of the label in the mixture) compared with 0.17×10^6 cpm without added mRNA. The background of radioactivity was not due primarily to protein synthesis, since the amount precipitable in the absence of added mRNA was only slightly decreased by inclusion of 0.3 mM cycloheximide in the reaction mixture (13) (result not shown).

Reaction mixtures primed with globin mRNA (9 S), late Ad2 cytoplasmic RNA, or uninfected KB cell cytoplasmic RNA, or with no added RNA, were fractionated by electrophoresis on sodium dodecyl sulfate–polyacrylamide slab gels (Fig. 2). Without added RNA several minor polypeptides were apparent (Fig. 2*c*). The 11,000-dalton polypeptide migrated with globin (Fig. 2*b*) and probably results from contaminating 9S RNA in the reticulocyte factor prepara-

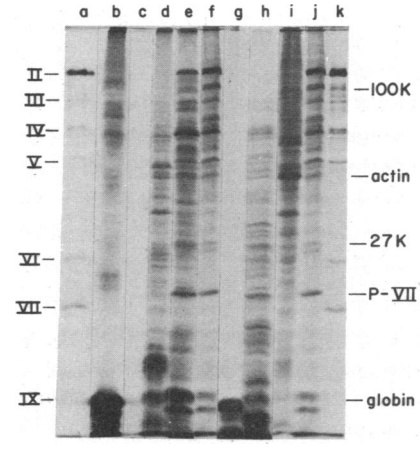

FIG. 2. Sodium dodecyl sulfate–polyacrylamide gel analysis, as previously described (3), of the products of *in vitro* protein-synthesizing systems programmed with Ad2 mRNA. The gel compared [^{35}S]methionine-labeled purified Ad2 proteins (*a* and *k*) with cell-free product. The mammalian system was programmed with (*b*) 0.5 μg of rabbit globin mRNA, (*c*) no added RNA, (*d*) 12 μg of unfractionated cytoplasmic RNA from uninfected KB cells, and (*e*) 12 μg of unfractionated cytoplasmic RNA from Ad2-infected KB cells. The wheat germ S-30 system (17) was programmed with (*g*) 0.5 μg of globin mRNA and (*h*) 12 μg of unfractionated cytoplasmic RNA from Ad2-infected cells. Also shown are the patterns obtained from [^{35}S]methionine-labeled (*i*) HeLa cells (1-hr pulse) and (*f* and *j*) Ad2-infected HeLa cells labeled 24–25 hr after infection. *Labels* on the *sides* indicate the migration of marker proteins and identified polypeptides (see *text*).

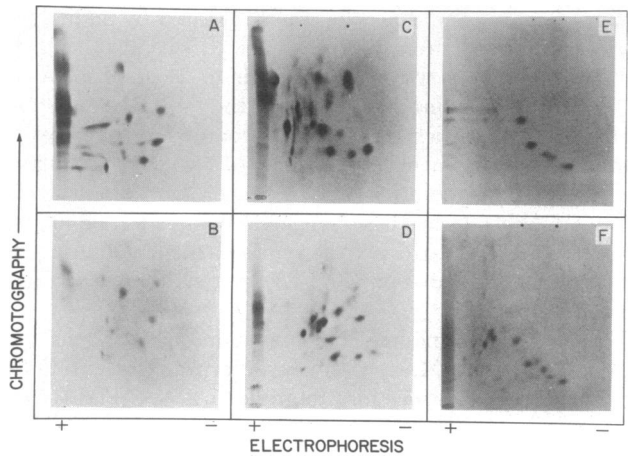

FIG. 3. Tryptic fingerprint comparison of *in-vitro*-synthesized Ad2 products with purified adenovirus virion components. Samples were prepared as described previously (3). Shown are fingerprints of (*A*) virus hexon, (*B*) *in vitro* hexon region, (*C*) virus fiber region, (*D*) *in vitro* fiber region, (*E*) *in vivo* major core precursor, (*F*) *in vitro* major core precursor.

tions. Cytoplasmic RNA from uninfected (Fig. 2*d*) and from Ad2-infected KB cells (Fig. 2*e*) stimulated the synthesis of complex but distinctly different polypeptides. In the case of the RNA from infected cells, a number of the polypeptides seen migrated with polypeptides found in Ad2-infected HeLa cells (Fig. 2*f* and *j*). In particular, there was comigration of polypeptides synthesized *in vitro* and most of the major Ad2 virion components, including hexon (II), penton base (III), fiber (IV), core (V), and the hexon-associated component (IX). Polypeptides corresponding to virion components VI and VII were not detectable among the products synthesized *in vitro*. However, *in vivo* these virion components are derived from precursor polypeptides (27K, P-VII) (3). Prominent cell-free products corresponding in size to each of the precursors of components VI and VII were synthesized. The identification of less prominent virus-specific proteins was hindered by a background of minor unidentified polypeptides. These probably resulted from premature termination of peptide synthesis, as has been observed with cell-free translation of encephalomyocarditis virus RNA (14).

An autoradiogram similar to that shown in Fig. 2*e* was scanned with a densitometer in order to obtain estimates of the proportion of each of the major Ad2 polypeptides synthesized *in vitro*. Compared to the total product fractionated on the gel (i.e., polypeptides larger than about 10,000 daltons), the hexon region contained 2%, the fiber region 12%, the core precursor region 5%, and the component IX region 10% of the [^{35}S]methionine. A whole-cell extract of Ad2-infected cells labeled 24–25 hr after infection had 17%, 6%, 6%, and 2% of the incorporated [^{35}S]methionine in these polypeptides, respectively.

That the major polypeptides synthesized *in vitro* in response to Ad2 mRNA migrated with Ad2 polypeptides on sodium dodecyl sulfate–polyacrylamide gel electrophoresis is strong evidence that the cell-free system synthesized authentic Ad2 polypeptides. This conclusion was verified by comparison of tryptic fingerprints of viral and cell-free synthesized polypeptides. Peptide patterns obtained from corresponding pairs of polypeptides synthesized *in vivo* and *in vitro* are compared in Fig. 3 for hexon (II), fiber (IV), and major core precursor (P-VII). There is correspondence between most of the major methionine-containing peptides in each pair. A similar correlation was apparent with the matching pairs of minor core (V) and component IX (data not shown).

The lack of complete correspondence of methionine-containing peptides between respective pairs of fingerprints is not surprising. Some of the bands seen on autoradiograms do not result from single polypeptides. For example, the fingerprint of fiber (Fig. 3*C*) is actually a fingerprint of two virion components (IIIa and IV) with nearly identical mobilities on gels. Furthermore, it has been shown that fiber protein is both glycosylated (15) and phosphorylated (16); however, such alterations may not occur *in vitro*. In fact, fiber protein synthesized *in vitro* moves slightly ahead of authentic fiber protein on sodium dodecyl sulfate–polyacrylamide gels.

Wheat Germ Translation System. The wheat germ S-30 system (17) programmed with Ad2 RNA incorporated in a 50-μl reaction mixture 1.0×10^6 cpm out of 20×10^6 cpm [^{35}S]methionine with a background of 0.17×10^6 cpm in the absence of added RNA. It produced much the same polypeptide pattern as the mammalian system (Fig. 2*h*). However, there was an obvious reduction in the proportion of larger molecular weight viral products (e.g., hexon), which could be due to RNase activity. For this reason we have concentrated on the mammalian system.

Size Classes of Ad2 mRNAs. Cytoplasmic RNA from Ad2-infected cells was fractionated by sedimentation through sucrose gradients. The RNA from each fraction was translated, and the resulting polypeptides were analyzed on sodium dodecyl sulfate–polyacrylamide gels (Fig. 4). The viral proteins synthesized *in vitro* could be distinguished by virtue of their migration with authentic Ad2 polypeptides. The mRNA species for each of the identifiable Ad2 proteins was found in a characteristic position, and was distributed in only a few fractions. The mRNA's for hexon (II), minor core (V), IX, and 11.5K proteins are about the size appropriate for the single polypeptides they generate (Table 1). The 27K protein and the 100K protein are encoded by 27S RNAs, one of which would be sufficiently large to encode both. The major

TABLE 1. *Comparison of the molecular weight of some virus-specific proteins with the apparent coding capacity of their mRNAs*

Protein			Messenger RNA	
Component		Molecular weight	Sedimentation coefficient (S)	Coding capacity (molecular weight of protein)
100K		100,000	27	165,000
27K		27,000	27	165,000
III	Penton base	70,000	25	145,000
II	Hexon*	120,000	23	120,000
P-VII	Core precursor*	20,000	21	103,000
IV	Fiber*	62,000	19	82,000
26K		26,000	19	82,000
V	Core-1*	48,500	16	62,000
IX	Hexon-assoc.*	12,000	9	15,000
11.5K		11,500	9	15,000

* Identified by fingerprinting.

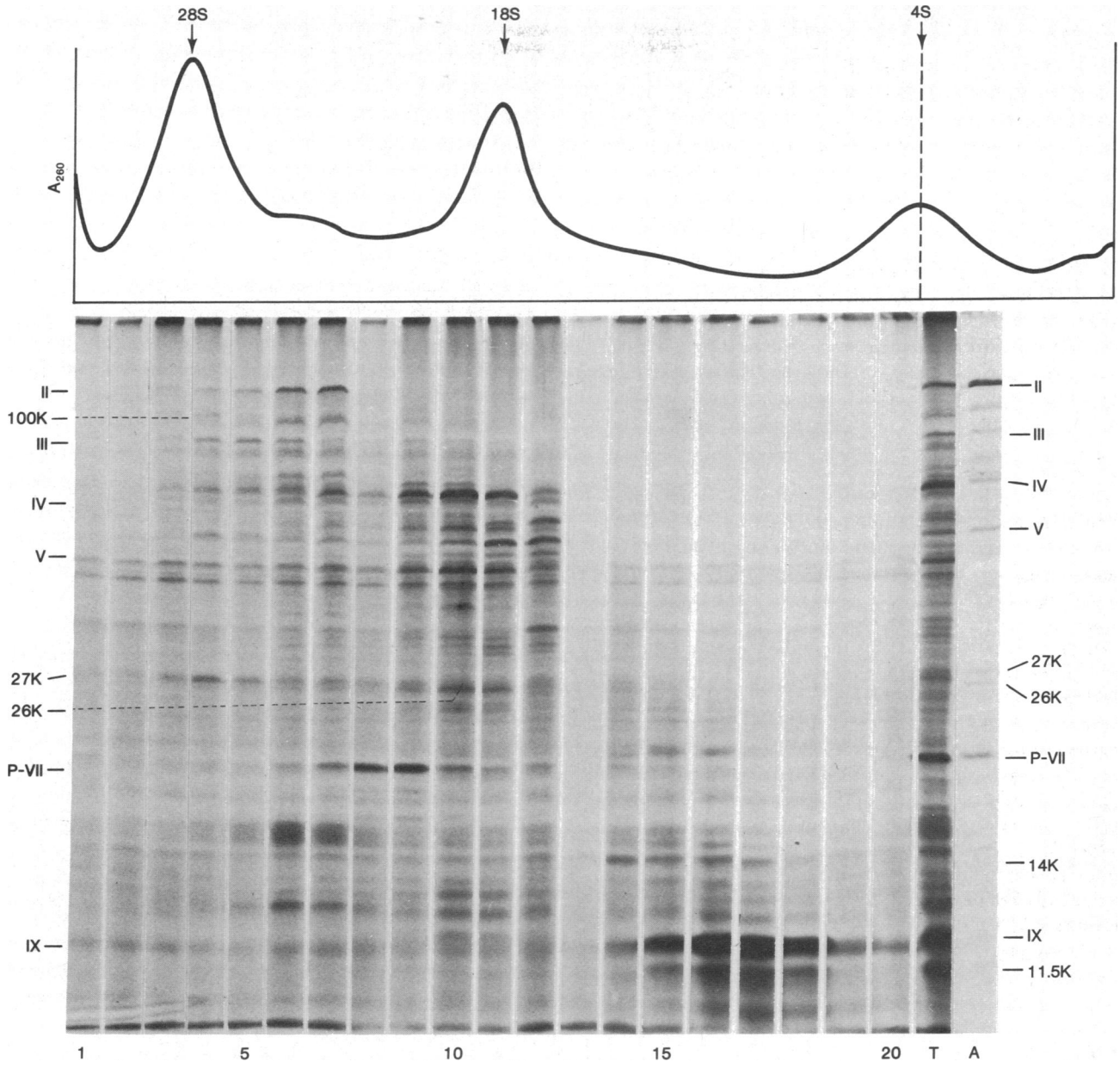

FIG. 4. Sodium dodecyl sulfate–polyacrylamide gel autoradiogram of the *in vitro* products programmed by fractionated Ad2 mRNA. RNA was fractionated on formamide-containing sucrose gradients according to the method of U. Lindberg (personal communication). RNA samples were prepared by incubation of 1 volume of RNA, 1 volume of 10× buffer (1 M LiCl, 0.05 M ethylenediaminetetraacetate, 2% sodium dodecyl sulfate, and 0.1 M Tris·HCl, pH 7.4), and 9 volumes of formamide (BDH Chemicals Ltd.) at 37° for 5 min, in order to denature the RNA. The RNA was then diluted with an equal volume of 1× buffer (the same buffer, diluted with 9 volumes of water); and 2.0 ml of solution containing 16 A_{260} units of RNA was loaded onto a 36-ml, 5–20% linear sucrose gradient made in 1× buffer containing 50% v/v formamide. Tubes were spun in the SW27 rotor (Spinco) at 27,000 rpm for 40 hr at 4°. Their contents were collected through a flow cell, and the absorbance at 260 nm was recorded. Each fraction (1.6 ml) was precipitated with 2 volumes of ethanol after addition of NaCl to a concentration of 0.5 M and of *Escherichia coli* ribosomal RNA to 15 μg/ml. After a second ethanol precipitation the remaining fluid was removed by brief lyophilization. Each fraction was dissolved in 50 μl of H_2O and stored at −20°. The mammalian cell-free system was programmed with 3–12 μl of RNA from each fraction and the products were analyzed on sodium dodecyl sulfate–polyacrylamide gels. The gel pattern is shown aligned with the optical density profile from the sucrose gradient; fraction numbers are given at the *bottom* of the figure. The cell-free product programmed by unfractionated cytoplasmic Ad2 mRNA (column *T*) and an *in vivo* labeled sample of Ad2-infected HeLa cell proteins (column *A*; same as Fig. 2*j*) are shown for comparison.

core precursor is synthesized predominantly from 21S RNA, approximately five times as large as required to synthesize a 20,000-dalton protein. Similarly, the penton base mRNA is two times too large. The fiber and 26K proteins are synthesized from 19S RNAs, of which one might be sufficient to encode both proteins.

DISCUSSION

We have shown that RNA from Ad2-infected cells can be translated in the cell-free system described by Schreier and Staehelin. More than half of the Ad2-specific products found in extracts of infected cells are synthesized. Five of these have been conclusively identified from their two-dimensional

tryptic fingerprints. Among the 10 products recognized are the principal virion components hexon (II), penton base (III), fiber (IV), core (V), and major core precursor (P-VII). The high-molecular-weight RNA fraction is the only component required from infected cells. Further, there is no stringent requirement for the source of the translational components, since the wheat germ system and the mammalian system give qualitatively similar results. In addition, Eron, Callahan, and Westphal (18) have shown that ascites cell S-30 extracts can translate Ad2 mRNA.

Analysis of the translation products made from Ad2 mRNA fractionated according to size shows that many of the viral proteins are translated from different mRNAs and therefore cannot all arise from a single large polypeptide precursor. The template RNAs for six of these proteins appear much larger than required to encode their amino-acid sequences alone. We cannot exclude that the apparent size of these mRNA species might result from RNA aggregation. However, this possibility seems unlikely, since each species sediments as a relatively homogeneous entity, and since several of the Ad2 mRNAs sediment at a rate predicted from the molecular weights of their protein products. Furthermore, qualitatively the same result has been obtained in separate experiments at different RNA concentrations with and without the use of formamide.

One possibility suggested by these data is that some Ad2 mRNAs are polycistronic in that they encode two or more independently initiated proteins. There are several examples of eukaryotic mRNA species that have excessive coding capacity for their known corresponding proteins (12, 19), but as yet there is no proven case of a eukaryotic polycistronic messenger. This possibility can be tested for Ad2 mRNA species, since we can ask if the synthesis of more than one polypeptide is directed by an RNA selected by hybridization to specific DNA fragments produced by endonuclease cleavage. Translation of such RNA should also permit detection of additional Ad2 gene products, particularly those expressed early and in transformed cells, and should lead to a physical mapping of these genes on the adenovirus DNA molecule.

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