The Origin and Destiny of Adenovirus Proteins

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Lytic infection of human cells by adenovirus proceeds by a temporal expression of genes. Classically two phases have been defined: an early phase, which includes events occurring before the onset of DNA synthesis (8 hr), and a late phase, including events whose existence depends on the onset of DNA synthesis. During the late phase of infection, host cell macromolecular synthesis is progressively inhibited so that eventually only virus-specific macromolecules are synthesized (for a review, see Philipson and Pettersson 1973).

Adenovirus DNA is transcribed in the nucleus of the infected cell by an α-amanitin-sensitive polymerase that is undoubtedly a host enzyme (Price and Penman 1972; Wallace and Kates 1972). At early times both DNA strands are transcribed, whereas at late times there is a bias towards transcription of the L strand (Green et al. 1970; Tibbetts et al. 1974). The sequences of RNA that appear in the cytoplasm constitute a specific subset of those found in the nucleus (Sharp et al.; Philipson et al., both this volume), implying that extensive processing of the RNA occurs. The details of this selection process and of the mechanism for the switch in strand bias remain obscure.

The processed RNA that does appear in the cytoplasm can be isolated as a ribonucleoprotein complex (Lindberg and Sundquist 1974). As described elsewhere in this volume (Sharp et al.; Philipson et al.; Craig et al.), nucleic acid reassociation techniques have been used to size the RNA species and to map the location of their corresponding DNA sequences. From such studies, the pattern of DNA expression is emerging.

Recently it has become possible to study viral RNAs functionally by assaying their ability to program the synthesis of their respective proteins in cell-free systems. A comparison of the cell-free products with those synthesized in vivo provides a method for the positive identification of specific messages.

Proteins in Infected Cells

At late times after infection, host protein synthesis is inhibited so that virus-induced proteins can be specifically labeled with radioactive amino acids (Bello and Ginsberg 1967). At least 22 adeno virus-specific proteins can be identified if total cell extracts are fractionated according to size on SDS-polyacrylamide gels (Table 1). Presumably these are gene products of adenovirus, but it is not ruled out that some could result from the induction of host genes. As shown in Figure 1 and reported previously (Anderson et al. 1973), 13 of these proteins have electrophoretic mobilities identical to proteins from similarly labeled purified virus particles. However, detailed examination of the kinetics of appearance of these proteins in infected cells makes it apparent that at least three proteins of the virus particles are cleavage products of larger precursor proteins.

Table 1. Adenovirus 2-induced Proteins

Band designation	MW	Relationship to virion
II	120,000	hexon
100K	100,000	nonvirion
III	85,000	penton base
71K	71,000	(DNA-binding-E_1-ICSP-3?)
IIIa	66,000	virion component
IV	62,000	fiber
IVa_1	60,000	virion component
IVa_2	56,000	virion component
50K	50,000	nonvirion
V	48,500	core
P-VI	27,000	precursor to VI
P-VIII	26,000	precursor to VIII
VI	24,000	hexon-associated
P-VII	20,000	precursor to major core (VII)
–	19,000	E_2 – glycosylated
VII	18,500	major core (AAP)
14K	14,500	
13.5K	13,500	
VIII	13,000	hexon-associated (appears during chase)
IX	12,000	hexon-associated
11.5K	11,500	
11K	11,000	(E_3?)
X	6500	virion component (appears during chase)
XI	6000	virion component (appears during chase)
XII	5000	virion component (appears during chase)

Molecular weights given here are apparent weights as determined by SDS-polyacrylamide gel electrophoresis and as previously described (Anderson et al. 1973). The three components (E_1, E_2 and E_3) described by Walter and Maizel (1974) have been included for the sake of completeness. The correlation of these components with those observed by us (Anderson et al. 1973), van der Vliet and Levine (1973), and Bablanian and Russell (1974) represent our present best guess; proof of these correlations has not yet been obtained. The sum of the molecular weights of the presumed independent late components (i.e., excluding VI, VII and VIII, but including 71K) is 860,000 daltons, or approximately 80% of the coding capacity of the virus. If 71K is also considered an early protein, the molecular weights of the known early components sum to 100,000 daltons or 10% of the coding capacity.

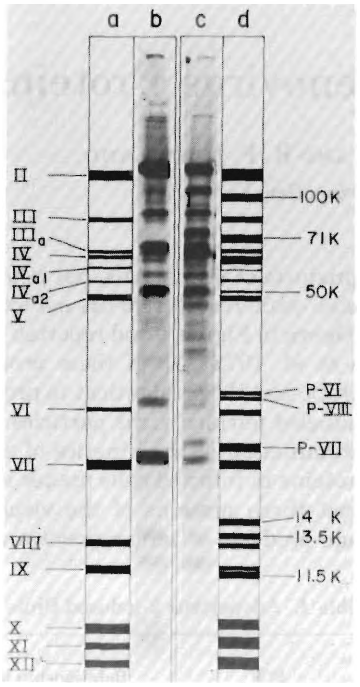

Figure 1. SDS-polyacrylamide gel autoradiogram of [^{35}S]-methionine-labeled purified virus and extract of infected whole cells. The gel contained 15% acrylamide and 0.087% bisacrylamide, as previously described (Anderson et al. 1973). Parts *a* and *d* are artist's drawings representing an idealized gel in which all the virion components *(a)* and the 22 virus-induced components *(d)* that we have observed are illustrated. Parts *b* and *c* are typical of the separations actually obtained: *(b)* [^{35}S]methionine-labeled purified virus; *(c)* a cell extract labeled with [^{35}S]methionine 18–19 hr p.i. (Reprinted, with permission, from Anderson et al. 1973.)

Figure 2 shows the pattern of proteins observed after 1-hour pulse-labeling with [^{35}S]methionine at progressively later times after infection. Synthesis of the late class of proteins can be seen by 13–14 hours after infection, when the shutoff of host cell protein synthesis becomes apparent. Comparison of these protein patterns to the pattern found with continuous labeling reveals that the major core protein (VII) is not found in the pulse-labeled samples. Instead, a protein (P-VII) 15 amino acids larger is seen. This is a precursor to the core protein, since radioactivity in P-VII can be chased into core protein and since P-VII gives a tryptic fingerprint identical to VII but with one additional methionine-containing peptide.

Pulse-chase experiments also show that proteins P-VI (27,000 daltons) and P-VIII (26,000 daltons) are unstable during the chase period. It is now clear from the work of Saborio et al. (unpubl.) that these proteins are precursors to virion components VI (24,000 daltons) and VIII (13,000 daltons), respectively. It seems most likely that these cleavages are an intimate part of viral assembly.

These are the only precursors that we can find, even using the shortest pulses possible. Hence we feel that large precursors of the picornavirus type (Jacobson and Baltimore 1968) do not exist in the case of adenovirus infection.

Several proteins have been found in infected cells that do not ultimately end up in virions. At late times proteins 100K, 71K, and 11.5K are obvious (Fig. 2). At early times it is very difficult to see virus-specific proteins against the background of host proteins. The data in Figure 2 suggest that the 71K protein is synthesized at earlier times than

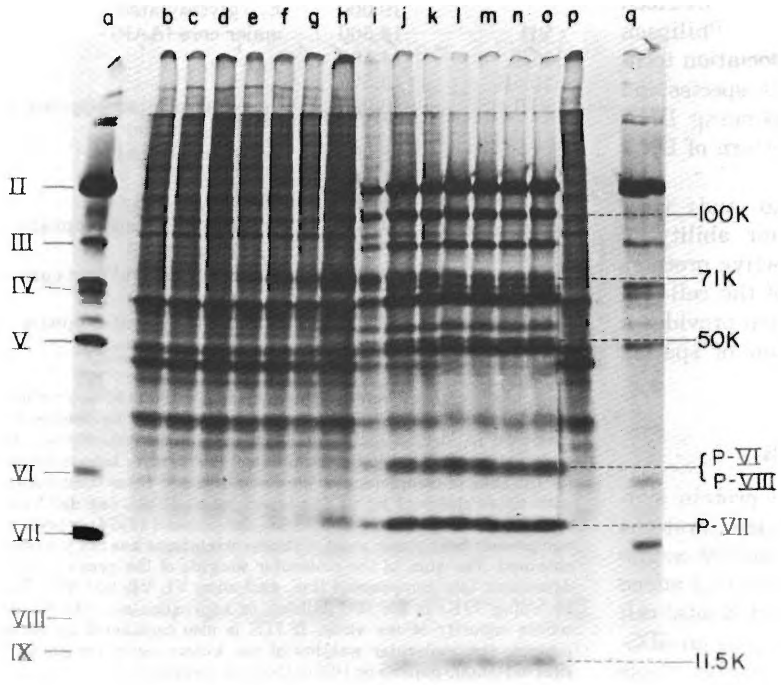

Figure 2. SDS-polyacrylamide gel autoradiogram of adenovirus 2-infected KB cells labeled at different times after infection. The gel contained 12.5% acrylamide and 0.1% bisacrylamide. A petri dish of infected KB cells was labeled for 1 hr in medium containing [^{35}S]methionine (20 μCi/ml) and carrier methionine (3 μg/ml). Samples were processed as previously described (Anderson et al. 1973). The sample order is: *(a)* purified [^{35}S]methionine-labeled virus; *(b)* mock-infected cells. Samples *c* through *o* are adenovirus 2-infected cells labeled 3–4, 5–6, 7–8, 9–10, 12–13, 13–14, 15–16, 16–17, 18–19, 20–21, 24–25, 27–28 and 30–31 hr after infection. *(p)* Mock-infected cells; *(q)* purified adenovirus 2. (Reprinted, with permission, from Anderson et al. 1973.)

the other adenovirus proteins, and it seems to be made in small amounts if the infected cells are kept in cytosine arabinoside to prevent transition to the late phase of infection (unpubl.).

Van der Vliet and Levine (1973) have functionally characterized a 72,000-dalton protein made early after infection and in the presence of cytosine arabinoside as a protein which binds to single-stranded DNA. It seems likely that this protein is the same as the 71K component described above; however, definitive proof is lacking. They have also described an adenovirus-specific 48,000-dalton DNA-binding protein, but higher resolution gels have subsequently shown this component to consist of at least four separable polypeptides of similar molecular weight (Rosenwirth and Anderson, unpubl.). Circumstantial evidence suggests that these may arise as degradation products of the 72,000-dalton protein.

Walter and Maizel (1974) find three adenovirus 2-induced early polypeptides. One of these (E_1) probably corresponds to the DNA-binding protein of van der Vliet and Levine (1973). A second (E_2) has a mobility similar to virion component VII but is glycosylated and thus is probably not related to the major core protein. A third component (E_3) migrates slightly ahead of virion component IX on SDS-polyacrylamide gels.

Bablanian and Russell (1974) have used a combination of guanidine and poliovirus to preferentially inhibit host cell protein synthesis in order to more readily detect early adeno-5 proteins. They find in this system, that, in the presence of cytosine arabinoside, polypeptides corresponding in mobility to ICSP-3 (64,000), core 1 (46,000), p-core 2 (equivalent to P-VII in adeno-2) and ICSP-5 (16,000) are preferentially synthesized. Thus these proteins are candidates for virus-coded early functions, but correlation of these adeno-5 proteins either with adeno-2 proteins or with specific early antigens, such as T or P, is not yet possible.

Current estimates from hybridization experiments say that 20% of the adenovirus DNA sequences are represented in cytoplasmic RNA (Fujinaga and Green 1970; Sharp et al., this volume) at early times after infection. This could encode at most 200,000 daltons of protein. At late times this climbs to 80% or about 900,000 daltons of protein. The sum of the molecular weights of the observed independent proteins at late times (i.e., all except E_2 and E_3) is 861,000 daltons, which therefore accounts for most of the expected viral expression. This conclusion assumes that none of the polypeptides listed in Table 1 is a fragment of any of the other polypeptides listed, except for those three pairs that have already been identified as being precursor and product. However, studies of the synthesis of proteins in infected cells do not provide direct proof of whether these proteins are virus coded.

Virus Assembly

Assembly of adenovirus occurs in the nucleus, necessitating transport of completed protein chains from cytoplasmic ribosomes through the nuclear membrane. A majority of the precursor proteins, P-VII, P-VIII and P-VI, labeled during a 3-hour pulse at 24 hours after infection, is found in the nucleus. Hence we think that the processing step is not involved in transport of the proteins into the nucleus. In fact, P-VII, P-VIII and P-VI proteins can be found in virus particles. We can follow the fate of the precursors in completed virions by purifying virus from cells labeled for 3 hours at 24 hours after infection, followed by chase periods of 0, 3, 6, 12, and 24 hours. The nonchased virus preparation, which had a normal density for virus of 1.34 g/ml in CsCl, has all three precursor proteins as well as their processed counterparts V, VII and VIII (Fig. 3). As the chase period increases, the radioactivity in all the precursors decreases. This shows that at least some of the precursor cleavage occurs only very late in the assembly—after the virion has assumed its final density. This raises the possibility that the cleavage enzyme might be a virion protein. However, attempts to demonstrate cleavage in vitro by incubation of virions have so far been inconclusive. In addition to our observations with complete virus particles, Ishibashi and Maizel (1974) and Sundquist et al. (1974) have reported that incomplete virus particles that lack DNA contain the precursor proteins.

All the results taken together suggest that the processing of the proteins may be an integral part of the late stages of viral assembly, but they do not rule out the possibility that the cleavages are trivial artifacts and not essential for production of infective virus. This question could be answered if particles could be prepared that had *only* unprocessed proteins. Caution must be exercised, however, since only one in 10–100 virus particles is infectious, so that looking at the bulk of the particles might be misleading. A number of different conditions do, however, alter the cleavage process. Incubation of infected cells at 42°C or in the presence of proflavin results in greatly reduced assembly of particles. Under these conditions the processing of P-VII is substantially reduced in both rate and extent. Also, in infected CV-1 cells assembly of adenovirus is very poor (Baum et al. 1972) and P-VII is not processed (unpubl.). However, in these cells synthesis of all virion proteins is reduced up to fivefold compared to synthesis in infected KB cells, and little if any fiber and P-VI proteins are made (Grodzicker et al. 1974). Nonetheless this case of abortive infection again shows a correlation between deficient assembly and a lack of processing of P-VII. The most we can say is that this circumstantial evidence suggests that cleavage of the precursors is involved in viral maturation.

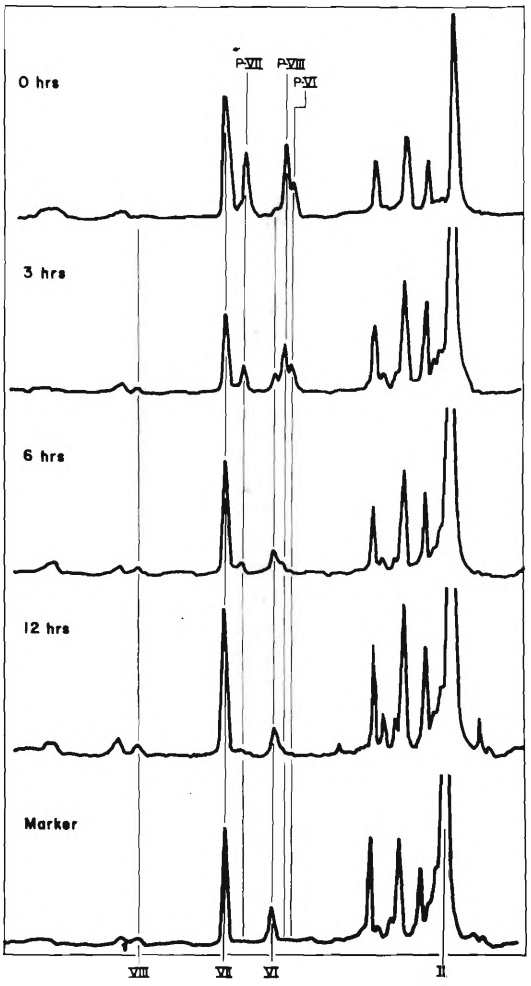

Figure 3. The components of young Ad2 virions. A culture of 4×10^8 cells was infected with 4×10^{10} PFU Ad2. Beginning at 26 hr p.i., the cells were labeled for 3 hr (1 mCi/100 ml of medium containing ~3 μg/ml methionine), followed by a chase period in medium containing 15 μg/ml nonradioactive methionine. After 0, 3, 6 and 12 hr of chase, samples were removed from the culture and frozen as cell pellets. When all samples had been collected, virus was purified from each sample by the standard method (Anderson et al. 1973), including freon extraction, sedimentation through CsCl onto a shelf, and equilibrium CsCl banding. Each sample was further purified by sedimentation through a low-salt sucrose gradient. Sufficient cold, purified virus was added at the beginning of purification to follow the virus band visually. All operations were done between 0 and 4°C. An aliquot of the purified virus was disrupted in SDS sample buffer and analyzed by polyacrylamide gel electrophoresis. A second aliquot was subjected to analysis by equilibrium CsCl density centrifugation. Virus from all four samples gave a single symmetric peak of density 1.34 g/ml with no significant amount of material banding elsewhere in the gradient.

Cell-free Synthesis of Adenovirus Proteins

Studies of the synthesis in vivo of virus-induced polypeptides have identified many of the proteins that are presumptive adenovirus gene products. Hybridization experiments, depending on the use of specific DNA fragments produced by restriction enzymes, are leading to a detailed picture of the species of viral mRNA found in the cytoplasm of infected cells. It should be possible to correlate these viral messages with their protein products by translating specific viral mRNAs in one of the cell-free protein-synthesizing systems recently developed (see Last and Laskin 1972). We have utilized the system first described by Schreier and Staehelin (1973) to analyze the products from each of several size classes of adenovirus message.

The incorporation of labeled amino acids into polypeptides in the fractionated mammalian cell-free protein synthesis system of Schreier and Staehelin is stimulated by cytoplasmic RNA isolated from KB cells late after adenovirus infection (Anderson et al. 1974). Under optimal conditions, a 50-μl reaction mix will incorporate 3×10^6 cpm of [^{35}S]methionine, out of a total input of 20×10^6 cpm [^{35}S]methionine, into acid-insoluble material. About 0.2×10^6 cpm is incorporated in the absence of added mRNA, and only a fraction of this is protein synthesis since the incorporation is only slightly depressed by cycloheximide.

The polypeptide products of cell-free synthesis were analyzed by polyacrylamide gel electrophoresis. An autoradiogram of [^{35}S]methionine-labeled polypeptides is shown in Figure 4. Identification of polypeptides is by comigration with disrupted adenovirus virions (Fig. 4a) and with extracts of infected cells labeled in vivo with [^{35}S]methionine (Fig. 4f). In the absence of added RNA, the major polypeptide synthesized is rabbit globin, but it is seen only in small amounts (Fig. 4b). Synthesis programmed by cytoplasmic RNA from uninfected cells (Fig. 4c) produced a complex pattern of polypeptides, resembling the pattern from uninfected cell extracts labeled in vivo (Fig. 4d). Cytoplasmic RNA from cells late after infection (Fig. 4e) programs the synthesis of the virion components hexon (II), penton base (III), fiber (IV), minor core (V) and the hexon-associated component (IX). In vivo, the virion components VI and VII (major core) and VIII are derived from precursor polypeptides (P-VI, P-VII and P-VIII) (Anderson et al. 1973; Saborio et al., unpubl.). The cell-free product contains polypeptides migrating with these three precursor polypeptides, but not with the virion components VI, VII and VIII. In addition, the cell-free product contains polypeptides that migrate with several other polypeptides (100K, 11.5K) seen in extracts of infected cells but not in the virions. Polypeptides corresponding to virion components IIIa, IVa$_1$ and IVa$_2$ and to the nonvirion polypeptide 50K may be present in the cell-free product, but they are not well enough resolved or present in large enough quantity to make certain identification possible.

RNA preparations isolated 18, 24 and 30 hours after infection all gave qualitatively similar polypeptide patterns.

The in vitro-synthesized polypeptides putatively

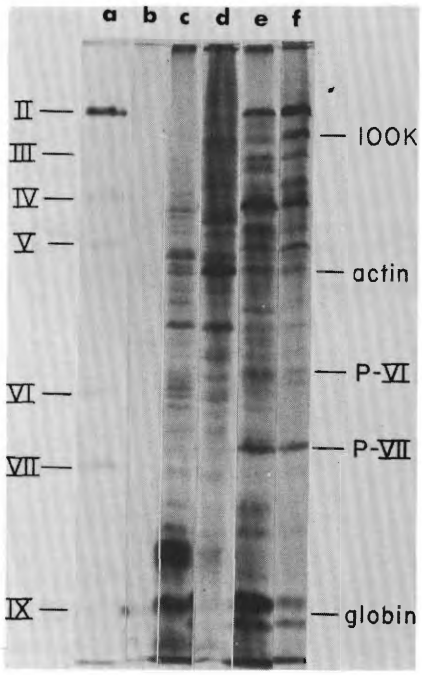

Figure 4. SDS-polyacrylamide gel analysis of the products of the mammalian cell-free protein synthesis system optimized for adenovirus RNA as previously described (Anderson et al. 1974). Identification of viral polypeptides is provided by *(a)* [^{35}S]methionine-labeled purified Ad2 virion proteins and *(f)* Ad2-infected HeLa cells labeled 24–25 hr p.i. Uninfected Hela cells (1-hr pulse of [^{35}S]methionine) are included for comparison *(d)*. The cell-free system was programmed with *(b)* no RNA, *(c)* 12 μg unfractionated cytoplasmic RNA from uninfected KB cells, and *(e)* 12 μg unfractionated cytoplasmic RNA isolated 30 hr after infection of KB cells with Ad2.

identified as hexon (II), fiber (IV), minor core (V), precursor to major core (P-VII) and component IX have been found to have two-dimensional tryptic peptide patterns very similar to those of the corresponding components from virions or from infected cells (Anderson et al. 1974).

We cannot draw any conclusions about the relative amounts of the adenovirus messages from these experiments, since it is not likely that all species are translated with the same efficiency and since we do not know the efficiency for any species. Qualitatively, however, we can say that the predominant cell-free products are the fiber (IV) and IX proteins. This contrasts with the fact that hexon is the major protein being synthesized in infected cells at late times. It is possible, though, that hexon is underrepresented in the cell-free products because it is the largest protein made and hence its synthesis will be most sensitive to aberrations of translation, such as premature ribosome termination or RNase attack on the messenger. This argument cannot be applied to a comparison of the amount of fiber product with the other adenovirus proteins of lower molecular weight. Hence the relative excess of fiber synthesis seems real, suggesting that its message is either more abundant than others or has a markedly greater frequency of translation in vitro. Purification of specific mRNA species is needed to answer this question.

Cell-free synthesis of adenovirus proteins has also been demonstrated by Eron et al. (1974) and Westphal et al. (this volume) and by Saborio et al. (unpubl.) using extracts from ascites cells. These groups have further characterized the cell-free products by immunoprecipitation, using antisera directed against purified virion components. We have also demonstrated the synthesis of the major virion components (identified by electrophoretic mobility) in an S-30 system derived from wheat germ (Anderson et al. 1974). Taken together, these results indicate that translation factors specific for adenovirus mRNA are not required for the synthesis of adenovirus proteins.

Size Classes of Adenovirus mRNA Programming the Synthesis of Specific Adenovirus Proteins

Distinct size classes of adenovirus mRNA, as assayed by hybridization to Ad2 DNA, have been reported both early and late in infection (Parsons et al. 1971; Parsons and Green 1971; Lindberg et al 1972). These different size classes of RNA appear to hybridize to different fragments of Ad2 DNA, so that they are presumably distinct populations of sequences (Craig et al., this volume). Since we now have a functional assay for specific mRNA species, we can determine the size distribution of each assayable mRNA. Late adenovirus RNA was fractionated by sedimentation through a sucrose gradient containing formamide. Each fraction of the gradient was translated, and the product was analyzed by polyacrylamide gel electrophoresis (Fig. 5). Messenger RNA for each of the ten virus-specific polypeptides previously identified was found in only a few adjacent gradient fractions. A variable number of additional bands were seen with each fraction of RNA. Some of these might be the product of remaining host mRNA, or of mRNA for as yet unidentified virus-coded proteins. Most likely, the majority of such bands represent the premature termination of the synthesis of larger polypeptides.

The mRNA for each of the adenovirus proteins is present in only a few adjacent gradient fractions, showing that the species are homogeneous and that gross aggregation is not a problem under these conditions. There are seven distinct size classes of Ad2 mRNA, each of which programs the synthesis of either one or two of the ten adenovirus-specific polypeptides that we identify among the cell-free products. Since many of the adenovirus polypeptides are translated from different mRNAs, they cannot all arise from a single polypeptide precursor, which is consistent with our failure to find such a precursor in vivo. Furthermore, these results show that, in terms of functional mRNA, distinct size classes of

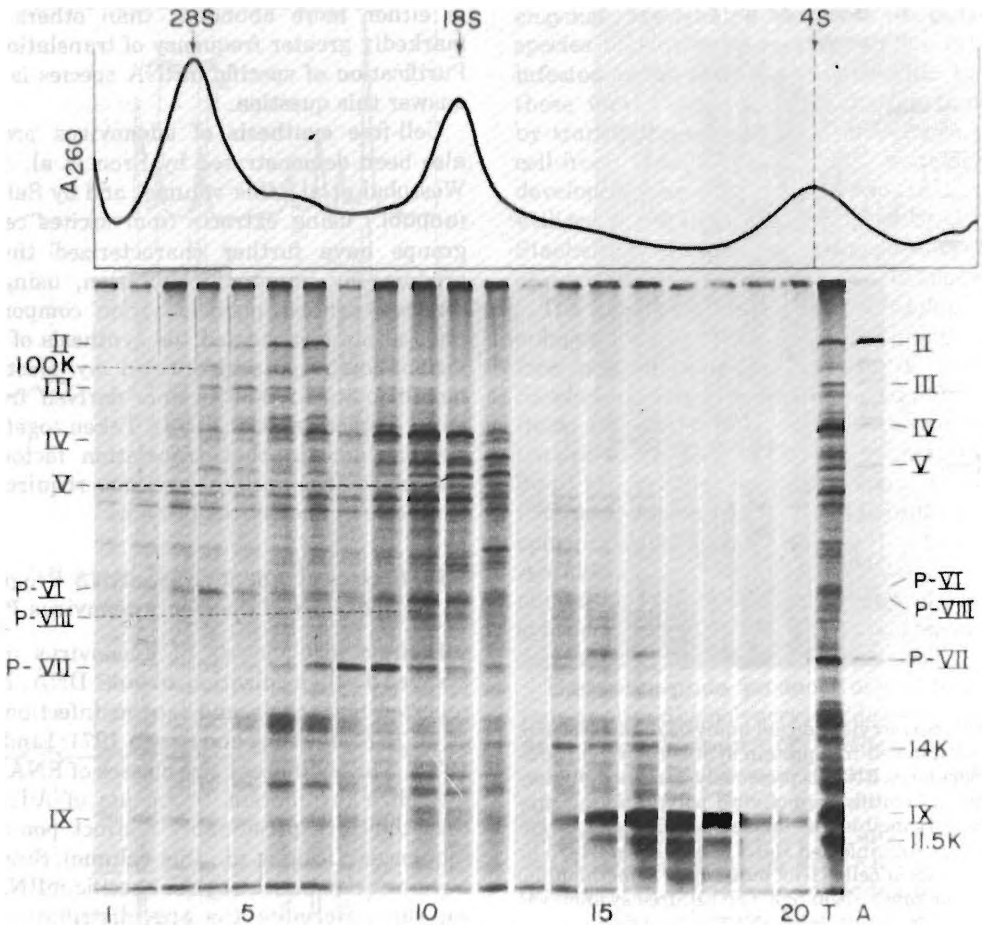

Figure 5. SDS-polyacrylamide gel autoradiogram of the in vitro products programmed by fractionated Ad2 mRNA. RNA was fractionated on formamide-containing sucrose gradients according to the method of U. Lindberg (pers. comm.). RNA samples were prepared by incubation of 1 vol. RNA, 1 vol. 10X buffer (1 M LiCl, 0.05 M EDTA, 2% SDS and 0.1 M Tris pH 7.4), and 9 vols. formamide (BDH Chemicals) at 37°C for 5 min in order to denature the RNA. The RNA was then diluted with an equal volume of 1X buffer, 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 1X buffer containing 50% v/v formamide. Tubes were spun in the SW27 rotor (Spinco) at 27,000 rpm for 40 hr at 4°C. Their contents were collected through a flow cell, and the absorbance at 260 mμ was recorded.

Each fraction (1.6 ml) was precipitated with two volumes of ethanol after addition of NaCl to a concentration of 0.5 M and of *E. 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 H_2O and stored at −20°C. The mammalian cell-free system was programmed with 3–12 μl RNA from each fraction, and the products were analyzed on SDS-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. 4f) are shown for comparison.

mRNA represent the expression of distinct viral genes.

The potential coding capacity of each mRNA can be estimated from its sedimentation constant. This is only approximate, since the formamide treatment used here would not be expected to destroy all RNA structure, so that sedimentation rate will not necessarily be determined by size alone. However, the approximate coding capacity of the mRNA and the size of the protein(s) coded by the mRNA are compared in Table 2. The mRNAs for hexon (II), minor core (V), IX and the 11.5K protein each have a coding capacity approximately equal to the size of the polypeptide product. On the other hand, the mRNAs for major core precursor (P-VII) and penton base (III) are respectively five and two times as large as required to synthesize these proteins. The P-VI and 100K proteins are encoded by mRNAs of the same size, and such an mRNA would have sufficient coding capacity for both polypeptides. The fiber and P-VIII proteins are also synthesized from mRNAs of the same size, and such an mRNA might have sufficient coding capacity for both polypeptides.

Cycloheximide can be used to inhibit the synthesis of late adenovirus RNA and to enhance the synthesis of early adenovirus RNA (Craig and Raskas 1974). RNA isolated from cells treated with cycloheximide, both uninfected and after adenovirus

Table 2. Comparison of the Molecular Weight of Some Virus-specific Proteins with the Apparent Coding Capacity of Their mRNAs

Protein			Messenger RNA		
component		MW (daltons)	S	nucleotides	calculated daltons of protein
100K	nonvirion component	100,000	27	4500	165,000
P-VI	precursor to VI	27,000	27	4500	165,000
III	penton base	85,000	25	4000	145,000
II	hexon	120,000	23	3200	120,000
P-VII	core precursor	20,000	21	2800	103,000
IV	fiber	62,000	19	2200	82,000
P-VIII	precursor to VIII	26,000	19	2200	82,000
V	core-1	48,500	16	1700	62,000
IX	hexon-associated	12,000	9	400	15,000
11.5K	nonvirion component	11,500	9	400	15,000
11K	early protein	11,000	22	3000	110,000

Sedimentation values (S) are approximate and were determined from Figs. 4 and 5, using the gradient fraction giving the largest amount of incorporation into the given component. Standards were the 28S, 18S and 4S RNAs present in the cytoplasm. Nucleotides per RNA species and the coding capacity in daltons of protein were calculated from the apparent S value; no correction was made for poly(A) content. The RNA species corresponding to most observed viral peptides were distributed in a few fractions of the gradients. The message for the early 11K component was more broadly distributed (6 fractions, 17–26S). Assuming each message represents an independent RNA species, the observed species of late message apparently account for 75% of the theoretical coding capacity of 34,500 nucleotides.

infection, was used to direct protein synthesis. The cell-free synthesis products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 6). The only obvious polypeptide found specifically in the product of infected RNA is one of 11,000 daltons.

The early RNA and uninfected RNA were fractionated by sedimentation through sucrose gradients, the various fractions were used to program cell-free protein synthesis, and the product was analyzed by polyacrylamide gel electrophoresis (Fig. 7). The 11,000-dalton polypeptide mRNA was found in a broad peak sedimenting at 20S. We have not detected a corresponding protein by analyzing whole cell extracts labeled at early times after infection. The E_3 protein of Ishibashi and Maizel (1974) could possibly correspond to this protein. We do not yet know if the early 11K protein is different from the 11.5K protein produced from late RNA (and found in vivo); however, they appear to have slightly different electrophoretic mobilities, and it is clear that the early and late species are encoded by different-sized mRNAs, 20S and 9S, respectively. If, however, the proteins are identical, then the synthesis or processing of the messenger must be different at early and late times.

Presumably other early adenovirus polypeptides are hidden by the background of host-specific protein synthesis seen in Figure 6. To study messages for these polypeptides, it will be necessary to select adenovirus-specific mRNA by hybridization to Ad2 DNA. These experiments are in progress.

The messages identified so far by these translation experiments are listed in Table 2, and from these data we can ask what portion of the genome known to be expressed at late times is accounted for by these RNA sequences. The total available sequence at late times is 80% of 35,000 base pairs, or 28,000 nucleotides.

If we assume that each of the messages listed in

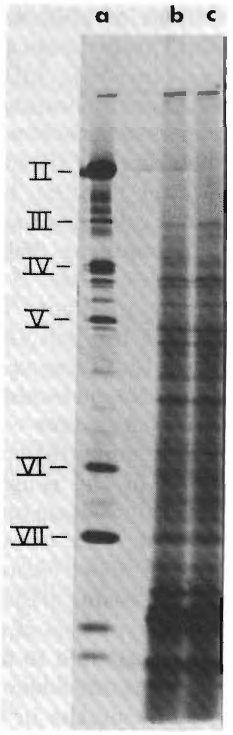

Figure 6. SDS-polyacrylamide gel analysis of the products of mammalian cell-free protein synthesis programmed with *(c)* 17 μg unfractionated cytoplasmic RNA from mock-infected KB cells and *(b)* 17 μg unfractionated cytoplasmic RNA from KB cells 18 hr after infection with Ad2. Both mock-infected and Ad2-infected KB cells were treated with cycloheximide at 12.5 μg/ml from 1–18 hr after infection.

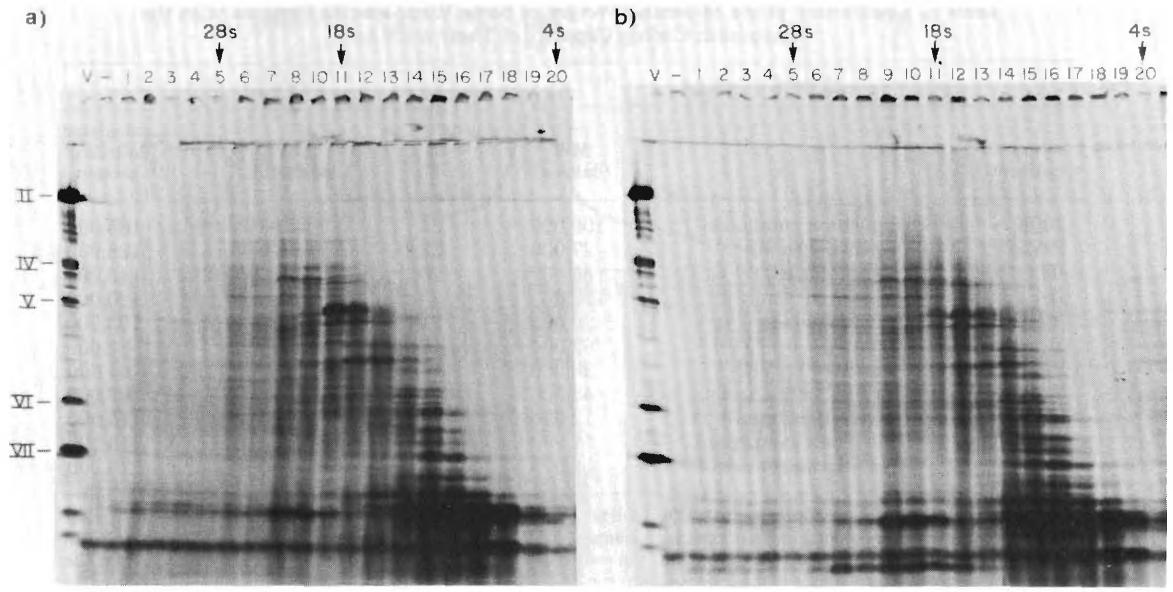

Figure 7. SDS-polyacrylamide gel autoradiogram of the products of cell-free protein synthesis programmed by fractionated RNA. The fractionation of the RNA on sucrose gradients is as described in the legend to Fig. 5. The RNA preparations used from mock-infected cells *(a)* and from cells early in Ad2 infection *(b)* are the preparations described in the legend to Fig. 6.

Table 2 is monocistronic, that is, encodes only the polypeptide identified with it in the table, then the late polypeptides we identify in our cell-free product total 512,000 daltons of protein, represented by 26,000 nucleotides. This estimate also assumes that poly(A) sequences contribute little to the size of the RNA and that those nucleotides not coding protein are not common to several mRNA molecules. The remaining polypeptides listed in Table 1 that are induced late after infection but have not yet been identified in our cell-free product total 348,000 daltons of protein, requiring 9500 nucleotides to encode their sequences. If the S values from the formamide-containing gradients truly reflect the molecular weights of the mRNAs, then the remaining possibility is that some pairs of proteins are encoded by single mRNA molecules.

If some adenovirus messages contain the information for more than one viral protein, they need not be polycistronic in the sense of *Escherichia coli lac* mRNA. Several products could be translated as a single unit and later cleaved. This seems unlikely, as discussed above. Another alternative is that cytoplasmic processing of the RNA is required to allow initiation of synthesis of a second protein product. It should be possible to demonstrate conclusively whether or not any adenovirus message is polycistronic by analyzing the in vitro translation products of individual messages purified by hybridization to restriction enzyme fragments of Ad2 DNA, and/or by gel electrophoresis.

Coupled Protein Synthesis from SV40 DNA

Another approach to the identification of viral gene products is to translate RNA made by in vitro transcription of the DNA. This has been particularly useful in studying bacteriophages. It has not yet been possible to do analogous experiments with the homologous DNA-dependent RNA polymerases in eukaryotic systems. However, in specific cases heterologous combinations can be useful. The *E. coli* RNA polymerase fortuitously transcribes the complete early strand of SV40 DNA (Lindstrom and Dulbecco 1972; Khoury and Martin 1972; Sambrook et al. 1972), yielding early mRNA and the sequence complementary to late RNA. With polyoma DNA as template, both strands can be transcribed (Kamen et al., this volume). By coupling this transcription to cell-free translation with all *E. coli* components, some major capsid protein of polyoma can be synthesized (Crawford and Gesteland 1973). The analogous SV40 product does not contain virion protein sequences, as expected from the fact that only early RNA is made. The SV40 products are mostly smaller than 40,000 daltons and are very heterogeneous, making interpretation difficult (Crawford et al. 1970).

In an attempt to get closer to homologous conditions, we have added viral DNA, *E. coli* RNA polymerase and extra triphosphates to the mammalian cell-free system used above.

Incorporation of amino acid into polypeptide was significantly simulated by SV40 DNA, but not by Ad2 DNA. A standard 50-μl incorporation mix incorporated 1.2×10^6 cpm of [^{35}S]methionine in the presence of 20 μg/ml SV40 DNA form I. Incorporation in the absence of DNA was 0.27×10^6 cpm. Analysis of the cell-free product by SDS-polyacrylamide gel electrophoresis (Fig. 8) revealed a number of polypeptides, none of which are synthesized in the absence of SV40 DNA. Disrupted

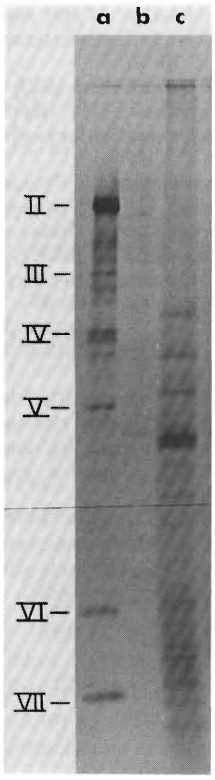

Figure 8. SDS-polyacrylamide gel autoradiogram of products of protein synthesis directed by DNA. A standard assay contained in 50 μl the following components: 0.25 A_{260} units ribosomal subunits, 5 μl pH 5 enzyme (containing 65 μg protein and 4 μg RNA), 12 μg 30–40% saturation $(NH_4)_2SO_4$ precipitated, DEAE-purified initiation factors, and 50 μg 40–70% saturation $(NH_4)_2SO_4$ precipitated initiation factors. The preparation of these components is described in Anderson et al. (1974). The other ingredients were 2.0 mM ATP, 0.6 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 20 mM creatine phosphate, 40 μg/ml creatine kinase, 60 μg/ml RNA polymerase (gift of Dr. B. Allet), 30 mM Tris-HCl pH 7.6, 100 mM KCl, 5.6 mM $Mg(OAc)_2$, 0.3 mM dithiothreitol, 30 μM each of 19 amino acids minus methionine, 2 μM [^{35}S]methionine at greater than 100 Ci/mM, and DNA as indicated below. Incubation was for 90 min at 35°C. The cell-free system was programmed with no DNA *(b)* and with 20 μg/ml SV40 form I DNA *(c)*. Disrupted adenovirus 2 is shown for a comparison of molecular weight *(a)*.

adenovirus is shown for a comparison of molecular weight. The cell-free product includes polypeptides of 70,000, 60,000, 50,000 and 40,000 molecular weight, as well as various smaller polypeptides. These proteins must now be compared with proteins from SV40-infected or -transformed cells, particularly the T and U antigens. The prediction that coupled synthesis from polyoma DNA should yield capsid proteins is being tested.

The coupled system of transcription and translation described here seems more promising than the preparation of SV40 cRNA followed by translation by mammalian components in a separate reaction. In the latter case, the largest polypeptide seen is 50,000 molecular weight and the predominant products are very small polypeptides (Lewis 1973). As yet none of these polypeptides have been identified.

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