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Vero cells injected with adenovirus type 2 mRNA produce authentic viral polypeptide patterns: Early mRNA promotes growth of adenovirus-associated virus

(microinjection/immunoprecipitation/parvovirus/polyacrylamide gel electrophoresis)

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ABSTRACT **Adenovirus type 2 mRNAs were injected via glass capillaries into Vero cells, a line of African green monkey kidney cells permissive for adenovirus growth. Polypeptides synthesized after injection were labeled with ^{35}S-labeled amino acids, precipitated with antiviral sera, and analyzed by polyacrylamide gel electrophoresis. Both early and late viral mRNAs give rise to authentic polypeptides. The *in vivo* translation of mRNAs can be measured as late as 24 hr after injection. The ability to analyze the protein products of microinjected mRNAs directly should greatly extend the applications of the procedure.**

Vero cells injected with early mRNA from adenovirus type 2 support the growth of adenovirus-associated virus, a defective virus that is dependent on adenovirus helper functions. This result demonstrates that a measurable biological activity, the ability to overcome the defectiveness of adenovirus-associated virus, resides in early adenovirus mRNA.

The initial stages of the adenovirus growth cycle are mediated by the concerted action of several early viral gene products (for review, see refs. 1 and 2). Our approach is to unravel the interplay of viral regulatory functions by examining individual circuits of the system under conditions that resemble as closely as possible genuine *in vivo* conditions. The microinjection technique (3) has been adopted for this purpose. With the help of this technique, it has become possible to transfer macromolecules such as DNA, RNA, and proteins via glass capillaries directly into animal cells growing on solid surfaces (3–8). In the previous studies, viral gene products expressed by the injected cells have often been characterized with the help of fluorescent antibody techniques. This approach provides information concerning the presence, in individual cells, of protein reacting with antibody. In our study, we will show that it is possible to display minute amounts of polypeptides, synthesized in a very limited number of injected cells, as bands in a polyacrylamide gel. This has enabled us to examine the size and the relative quantities of specific adenovirus gene products made *in vivo* in response to injected mRNAs. Together with the previously used fluorescent staining method, our procedure of detailed analysis of proteins synthesized in response to injected nucleic acid is expected to greatly extend the applicability of the microinjection technique to a wide variety of problems in eukaryotic biology.

It was important for us to show that adenovirus mRNA introduced by microinjection into cultured cells exhibited biological activities comparable to those present during genuine infection. To this end, we designed an experiment addressed to a current problem in Parvovirus research. Adenovirus-associated viruses (AAVs), members of the Parvovirus group, require adenovirus helper functions for their own replication (9, 10). The nature of the defect in these viruses is unclear. Work with temperature-sensitive mutants of helper adenovirus has been ambiguous in that it has implicated functions acting both at early (11–13) and at late stages (13) of AAV development. We have examined the adenovirus helper effect directly by injecting early adenovirus type 2 (Ad2) mRNA into permissive cells infected with AAV2 particles. Our results show unambiguously that early Ad2 mRNA is sufficient for AAV2 propagation.

MATERIALS AND METHODS

Cell Cultures. The Vero line of African green monkey kidney cells, permissive (14) for Ad2, was propagated in monolayer culture in minimal essential medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics (penicillin at 50 IU/ml, streptomycin at 50 μg/ml). Cells to be injected were grown in islands of up to 800 cells by placing small drops of a trypsinized cell suspension at various marked locations in a 6-cm plastic petri dish. Large drops of medium in the same petri dish provided the required humidity. The cells were allowed to attach to the surface for 30 min in the incubator. After this, fresh medium was added to the petri dish. Infection with AAV2 (5 tissue culture infectious units per cell) followed published procedures (15).

RNA Preparations. Extraction of early and late cytoplasmic RNA from KB cells infected with Ad2, enrichment for poly(A)-containing RNA sequences, and storage of the purified products have been described (16, 17). For the preparation of early RNA, cells infected with 10^4 Ad2 virions per cell were grown for 10 hr in the presence of 25 μg of cycloheximide per ml (18). We have characterized early RNA extracts of this type by electron microscopy and have found them to be devoid of late RNA, except for small amounts of truncated late RNA sequences (19). Both early and late RNA preparations were free of viral DNA, as judged by electron microscopy under conditions that distinguish single-stranded from double-stranded nucleic acid (20).

Cell-Free Translation. We used a Krebs II ascites lysate as described (14), except for two modifications. The lysate was pretreated with micrococcal nuclease to destroy endogenous mRNA (21), and spermidine was added to the translation assays at a concentration of 1 mM to enhance the synthesis of Ad2 proteins (22). Each assay (30 μl) contained 1 μg of total exogenous RNA, estimated by absorbance at 260 nm.

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Abbreviations: AAV, adenovirus-associated virus; Ad2, adenovirus type 2.

Microinjection. By the procedure of Graessmann *et al.* (3), RNA, dissolved at 1 mg/ml in 10 mM Tris·HCl (pH 7.5), was transferred into the cells with the help of glass capillaries attached to a micromanipulator. We calculate that between several hundred and several thousand viral mRNA molecules were injected into each cell. This rough estimate is based on the injection volume, cited as 10^{-10}–10^{-11} ml (3, 23), an RNA size range of 1–4 × 10^3 nucleotides (24, 25), and the fraction of viral sequences present in the RNA extracts. In RNA preparations such as ours, viral RNAs constitute a high percentage of all newly synthesized RNA. For early RNA, the cited value is 30% (26), for late mRNA 80% (18). However, despite the removal of most of the ribosomal RNA during oligo(dT) column chromatography, the newly synthesized sequences, identified by radioactive label, are likely to constitute only a fraction of the total RNA in the preparations. Our calculation of the amount of viral RNA injected into the cells is based on a conservative weight estimate of 1% viral RNA in the early and 10% in the late RNA preparation. This estimate is based on an approximate count of the number of hybridized compared to unhybridized RNA molecules visualized in the electron microscope after exhaustive hybridization to an excess of viral DNA.

***In Vivo* Labeling of Proteins.** For short-term labeling, medium without methionine and cysteine was supplemented with 0.5 mCi each of [^{35}S]methionine and [^{35}S]cysteine per ml (500–900 Ci/mmol, New England Nuclear; 1 Ci = 3.7 × 10^{10} becquerels), as well as dialyzed serum and antibiotics. In addition, unlabeled methionine and cysteine (50 μM each) and 0.25 μg of amphotericin B per ml (Fungizone, GIBCO) were added if the labeling period exceeded 2 hr. A 20-μl droplet of labeling medium was placed over the cell island after a ring was dried around the cells with a sterile cotton-tipped applicator.

Immunoprecipitation. After labeling, cell islands were repeatedly washed with phosphate-buffered saline. Cells were harvested in a 20-μl droplet of lysis buffer (0.5% Nonidet P-40/0.15 M NaCl/5 mM EDTA/50 mM Tris·HCl, pH 7.5). The lysis buffer was confined within a circle drawn around the cell island with a wax pencil. Before immunoprecipitation, the lysate was frozen and thawed twice. The following antisera were used in this study. (*i*) Anti-late serum was derived from rabbits injected with purified Ad2 coat proteins. This antiserum precipitates hexon, fiber, and, to a lesser degree, 100,000-dalton polypeptide and penton base. (*ii*) Anti-early serum was raised in sheep by injecting a lysate of KB cells harvested 8 hr after infection with Ad2 in the presence of 40 μg of cytosine arabinoside per ml. The serum was absorbed with a lysate of uninfected KB cells. The serum is directed mainly against the E 72,000-dalton polypeptide and also precipitates several proteins of uninfected cells. (*iii*) Anti-AAV serum was made in a rabbit injected with AAV2 virions that had been banded three times in CsCl gradients. The serum was enriched in immunoglobulin G by ammonium sulfate fractionation. The serum precipitates AAV2 virion proteins and also reacts weakly with Ad2 hexon. Immunoprecipitations were conducted in a volume of 100 μl, containing 20 μl of an appropriate dilution of antiserum and from 50 to 250 × 10^3 trichloroacetic-acid-precipitable cpm of cell lysate. After 1 hr of incubation at room temperature, antigen–antibody complexes were absorbed with 200 μl of a 10% (vol/vol) suspension of *Staphylococcus aureus* (Cowan I strain, Enzo Biochem) for 20 min at room temperature (27). After thorough washing (27), antigen was eluted by suspending the bacterial pellet in 20 μl of gel sample buffer [2% (wt/vol) NaDodSO$_4$/0.3 M 2-mercaptoethanol/20% (vol/vol) glycerol/0.002% bromophenol blue/0.1 M Tris·HCl, pH 7.5] and heating at 100°C for 5 min. The sample was centrifuged for 3 min at 10,000 × g_{av}, and the supernatant was analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Cell extracts, cell-free translation products, or immunoprecipitates were subjected to electrophoresis through polyacrylamide gels in the presence of NaDodSO$_4$ as described (14). After they were dried, gels were fluorographed (28) and exposed to Kodak XR1 film.

RESULTS

The messenger qualities of our Ad2 RNA preparations were tested in a protein-synthesizing system derived from Krebs II ascites cells. The cell-free translation products are shown in Fig. 1 as polypeptide bands separated by gel electrophoresis. The bands correspond well to the viral gene products known to be encoded by the early (30–32) and late (16, 33) Ad2 RNAs. The polypeptides specified by our early Ad2 mRNA preparation include major bands of E 72,000 daltons, E 19,000 daltons, and E 11,000 daltons. Late products include the main structural components of the Ad2 virion and the 100,000-dalton protein.

Assured that we had preparations of active mRNA at hand, we injected late Ad2 mRNAs into the cytoplasm of cells susceptible to lytic infection. At various times after injection, the cells were exposed to medium containing [^{35}S]cysteine and [^{35}S]methionine. The patterns of polypeptides labeled after the injection of late Ad2 mRNA are seen in Fig. 2. Lanes 1 and 10 contained Ad2 marker polypeptides. The control (lane 2) showed a total cell extract of uninjected cells. A comparison of this control to the total cell extract of cells injected with the late Ad2 mRNA (lane 4) revealed bands of late Ad2 proteins, notably those of polypeptides II (hexon), III (penton base), and IV (fiber) plus several host-cell polypeptides. An antiserum directed against a number of late Ad2 proteins was used to precipitate virus gene products synthesized at various times

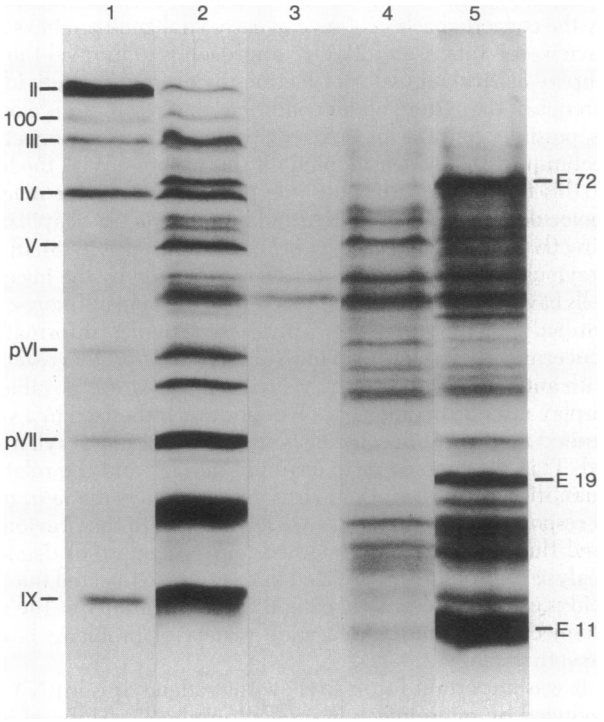

FIG. 1. Polypeptides derived from cell-free translation of early and late Ad2 mRNAs, separated by electrophoresis through a 20% polyacrylamide gel. Lane 1, late marker (total cell proteins, labeled with [^{35}S]methionine late after Ad2 infection) (16, 29). Lanes 2–5, cell-free translation products: lane 2, late RNA; lane 3, no RNA added; lane 4, RNA from uninfected cells; lane 5, early RNA. In all figures, individual Ad2 polypeptide bands are identified in the margins (arabic numbers are kilodaltons).

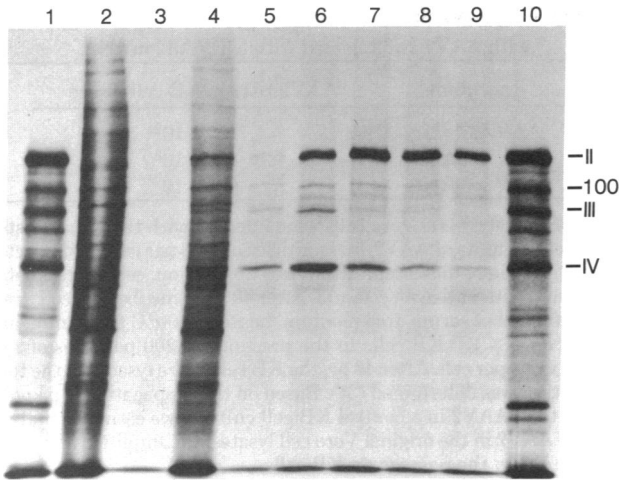

FIG. 2. Polypeptides from cells injected with late Ad2 mRNA. Anti-late serum was used for immunoprecipitation. Polypeptides were electrophoresed through a 13% polyacrylamide gel. Lanes 1 and 10, late marker (see legend of Fig. 1). Lanes 2–4, polypeptides labeled 3–5 hr after injection: lane 2, total cell extract of cells injected with buffer alone; lane 3, same, immunoprecipitated; lane 4, total cell extract of cells injected with late Ad2 mRNA. Lanes 5–9: immunoprecipitates of cells injected with late Ad2 mRNA and labeled 1–3 hr (lane 5), 3–5 hr (lane 6), 7–9 hr (lane 7), 11–13 hr (lane 8), and 15–17 hr (lane 9) after injection. Equivalent amounts of cell lysate ($\approx 5 \times 10^4$ cpm) were used for each immunoprecipitation.

after mRNA injection. The antiserum was quite specific and did not react with host-cell proteins (lane 3). However, as soon as 2 hr after injection, several Ad2 polypeptides, including II, 100,000 dalton, III, and IV, appeared in the immunoprecipitate (lane 5). A time course (lanes 5–9) showed that the rate of *in vivo* synthesis was different for individual Ad2 polypeptides. This was most conspicuous when the hexon and fiber bands were compared. We cut out these bands from the gel and determined their radioactivity in order to quantitate the result (not shown). Clearly, the maximum rate of hexon synthesis occurred several hours after that of fiber.

Similarly, early Ad2 mRNA was injected and proteins made *in vivo* in response to this RNA were precipitated with an antiserum directed mainly against the major early protein, E 72,000 daltons. Fig. 3, lane 1, shows the E 72,000-dalton polypeptide contained in the immunoprecipitate. Vero cells are permissive for Ad2 growth and synthesize the usual set of early viral proteins during lytic infection. Our early RNA preparation directed the cell-free synthesis of several virus-specific proteins in addition to E 72,000 daltons. This led us to assume that viral proteins other than E 72,000 daltons were expressed as well in cells injected with early RNA. We proceeded to examine whether the cells injected with early Ad2 RNA express the functions required to promote the growth of AAV2. Cells infected with AAV2 and injected with the early RNA were labeled with radioactive amino acids for an extended period of time. Extracts of these cells were tested for the presence of AAV2 gene expression by using an antiserum directed against the AAV2 virion proteins. The results are presented in Fig. 4. Flanked by late markers, a number of lanes show the polypeptides immunoprecipitated by the antiserum. Against a background of host polypeptides, lane 2, a positive control, displays the polypeptides precipitated from a cell coinfected with AAV2 and Ad2 helper virus. The major band of polypeptide C, migrating close to the Ad2 coat polypeptide IV (fiber), plus the two minor bands A and B are all characteristic for AAV2 (34). In addition, the antiserum precipitated a small amount of Ad2 coat protein II (hexon). This hexon band appears

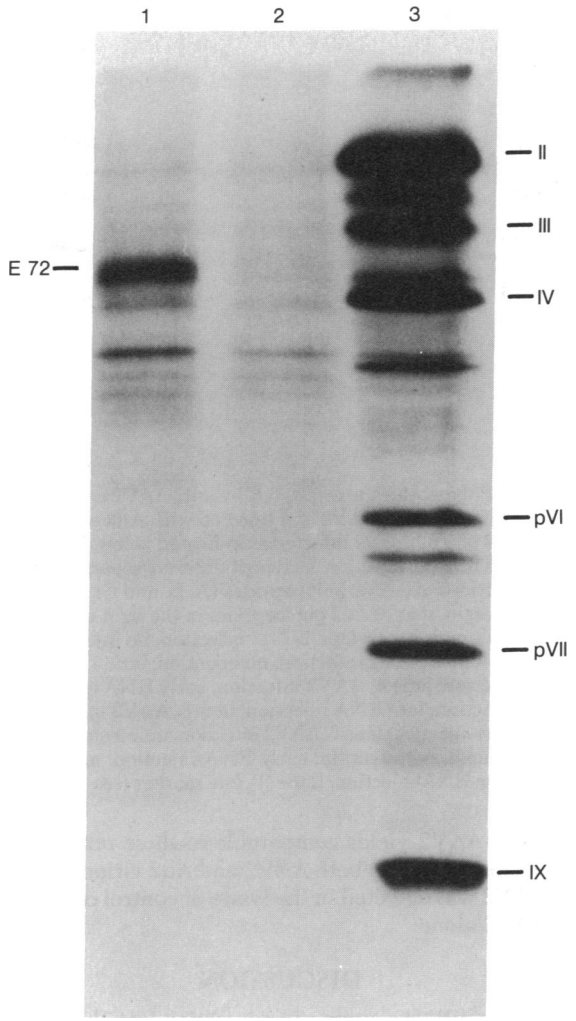

FIG. 3. Polypeptides of cells injected with early Ad2 mRNA. Anti-early serum was used for immunoprecipitation. Polypeptides were separated by electrophoresis through a 20% polyacrylamide gel. Lane 1, immunoprecipitate of cells injected with mRNA and labeled 4–8 hr after injection; lane 2, same, with buffer alone for injection; lane 3, late marker (see legend to Fig. 1).

also in precipitates of cells infected with Ad2 alone (lane 1), injected with Ad2 late RNA (lane 9), or infected with AAV2 and injected with Ad2 late RNA (lane 5). The important information is contained in lane 4. Immunoprecipitates of cells infected with AAV2 and injected with early Ad2 RNA clearly contain polypeptide C of AAV2. No trace of that polypeptide can be detected in cells not infected with AAV2 but injected with early RNA (lane 8), in cells infected with AAV2 and injected with RNA from uninfected cells (lane 7), in cells infected with AAV2 but not injected with any RNA (lane 3), or in cells infected with AAV2 and injected either with Ad2 late RNA (lane 5) or a mixture of early and late RNA (lane 6). A repeat experiment (not shown) demonstrated that actually all three AAV2 structural polypeptides were synthesized in cells infected with AAV2 and injected with Ad2 early RNA.

Not only were AAV2 proteins expressed in these cells, but infectious AAV2 was produced at levels comparable to usual infection schemes. This is shown in the experiment of Table 1. An island of Vero cells was infected with AAV2, injected with early Ad2 RNA, and incubated for a time sufficient to allow for AAV2 maturation. The lysate of these cells was passaged once on human KB cells to amplify the expected AAV2 yield. As seen in Table 1, injection of early Ad2 RNA resulted in the pro-

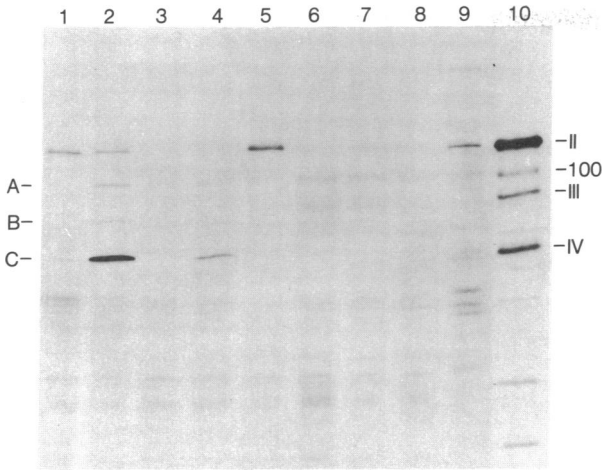

FIG. 4. Polypeptides precipitated by anti-AAV2 serum, derived from cells infected with AAV2 and injected with Ad2 mRNAs. Cells were injected and 3 hr later infected as indicated below. Labeling was from 13 to 23 hr after injection. Polypeptides were separated on a 13% gel. The positions of AAV2 polypeptides (A, B, and C) are indicated in the left margin, that of Ad2 polypeptides in the right margin. Lanes 1–9, immunoprecipitates: lane 1, Ad2 infection, no injection; lane 2, Ad2 infection plus AAV2 infection, no injection; lane 3, AAV2 infection, no injection; lane 4, AAV2 infection, early RNA injection; lane 5, AAV2 infection, late RNA injection; lane 6, AAV2 infection, early and late RNA injection; lane 7, AAV2 infection, uninfected cell mRNA injection; lane 8, no infection, early RNA injection; and lane 9, no infection, late RNA injection. Lane 10, late marker (see legend to Fig. 1).

duction of AAV2 yields comparable to those obtained from double infections with both AAV2 and Ad2 virions. No infectious AAV2 was detected in the lysate of control cells infected with AAV2 alone.

DISCUSSION

Adenovirus mRNAs, upon microinjection into the cytoplasm of uninfected cells, synthesize polypeptides that closely resemble those observed during genuine infection. Moreover, early Ad2 mRNAs help AAV2 to express its structural polypeptides in the absence of Ad2 and thus exhibit a measurable biological function inside the injected cell.

We have analyzed the proteins made *in vivo* in response to injected mRNA by $NaDodSO_4$/polyacrylamide gel electrophoresis. The method is convenient and reliable. It provides information concerning the size, relative quantity, and rate of synthesis of individual polypeptides. About 500 cells, injected with picogram amounts of RNA and labeled with 20 μCi of ^{35}S-labeled amino acids, suffice for one experiment. For instance, in Fig. 2, we measure about 1 cpm of an individual, immunoprecipitated polypeptide per injected cell. We are confident that the analysis could be scaled down to even smaller numbers of cells, but because we routinely inject about 600 cells in 1 hr, the above procedure was considered adequate for our purposes.

Cytoplasmic mRNAs of animal cells have been noted for their longevity (23, 36). Their stability is certainly reflected by that of the Ad2 mRNAs used in our study. If we consider, for example, that a single nucleolytic cleavage in the 4000-nucleotide chain of hexon mRNA is quite likely to prevent the synthesis of a full-size gene product, it is reassuring to observe active synthesis of the 120,000-dalton polypeptide 16 hr (Fig. 2) or even 24 hr (data not shown) after injection. It would appear that the rate of Ad2 protein synthesis falls precipitously later after injection. We note, however, that equivalent amounts of labeled cell protein were immunoprecipitated at each time point. Cells

Table 1. Production of infectious AAV2 in Vero cells infected with AAV2 and injected with early Ad2 mRNA

Inoculum	AAV2 titer in KB cell lysate
AAV2	$< 10^2$
AAV2 + Ad2	1.1×10^8
AAV2 + RNA	6×10^7

Approximately 300 Vero cells were infected with the viruses listed (5 infectious units of AAV2, 100 particles of Ad2 per cell) and injected with RNA where indicated. After virus absorption, cells were treated for 30 min with anti-AAV2 serum. After 40 hr of incubation, cells were lysed in 25 μl of serum-free medium, heated at 60°C for 15 min, and plated on 2×10^5 KB cells in the presence of 200 particles of Ad2 helper virus per cell. After 48 hr, the KB cells were lysed and the total AAV2 titer was determined (35). Based on the propagation of a known quantity of AAV2 in a control KB cell culture, we estimate that the titer of AAV2 in the original Vero cell lysates was amplified about 1.25×10^4-fold by the passage on KB cells.

appear to continue to actively synthesize their own polypeptides and, from the observation of mitoses, we conclude that some cells even go on to divide after injection. Therefore, both an actual increase in the synthesis of host proteins and a concomitant decrease in the synthesis of viral proteins are likely to contribute to the decline of hexon and fiber bands observed late after injection (Fig. 2).

The observed difference in the rates of fiber compared to hexon synthesis, confirmed in repeat experiments, may be a direct result of microinjection, because it is not observed during genuine infection (37). This phenomenon may reflect a rate-limiting step, determined either by diffusion of RNAs of different size through the cytoplasm (38) or by differential unfolding of their secondary structures.

Continued host-cell protein synthesis after the injection of late Ad2 mRNA leads to complex gel patterns, showing both viral and cellular polypeptides (Fig. 2, lane 4). In contrast, extracts of cells harvested late after genuine infection with Ad2 show almost exclusively viral polypeptides (Fig. 2, lane 1). The shutoff of host protein synthesis late after Ad2 infection has been attributed to a competition between host mRNAs and excess Ad2 mRNAs at the ribosome level (39). There are several ways to explain why we fail to observe this shutoff in our injected cells. First, only a fraction of cells may have been involved in the synthesis of Ad2 proteins. This is unlikely because all cells received mRNA and fluorescent staining (not shown) indicated that most, if not all, of the injected cells produced viral antigen. Second, not enough RNA may have been injected to compete effectively with host mRNAs. This argument may be valid. Our calculation of the amount of injected RNA (see *Materials and Methods*) is not sufficiently accurate to determine whether it matches the number of viral mRNAs presumed to be present in the infected cell (40). Our attempts to use more concentrated RNA preparations for injection did not meet with success. Rather, it appeared that the concentrate was frequently extruded by the cell, in what appeared to be spherical vesicles, within a minute after injection. Our assays did not reveal any synthesis of viral products in these cells either. Third, genuine infection may favor translation of late mRNA over that of host mRNA or may preferentially degrade host mRNA by use of unknown factors. If so, these factors were not, or not sufficiently, active in the cells injected with late Ad2 mRNA.

Our finding that cells injected with early Ad2 mRNAs are able to promote the growth of AAV2 not only demonstrates a measurable biological activity of the injected RNA, but contributes to the understanding of the defectiveness of the AAVs. Because careful analysis (19) has failed to detect the presence of late mRNA in our preparation of early Ad2 mRNA, we

conclude that early Ad2 gene expression suffices to provide the helper effect. The results obtained with early temperature-sensitive mutants of adenovirus (11–13) imply that proteins, rather than the mRNA itself, mediate the helper effect. Injection of individual early mRNA species is expected to determine whether subsets of early Ad2 mRNA are sufficient to provide the helper effect, as suggested (41). Our finding that the adenovirus helper effect is restricted to early viral genes does not necessarily imply that the functions involved are all normally active at early stages of adenovirus development. In fact, certain adenovirus mutants temperature sensitive in virion assembly are also impaired in promoting AAV growth at the restrictive temperature (13). The possibility exists that there are early adenovirus gene products that act late or that have dual functions both early and late in the adenovirus growth cycle.

Our preparation of late Ad2 mRNA did not provide helper functions for AAV2 growth. Late Ad2 mRNA contains early RNA sequences as well (42). However, the late mRNAs present in high copy numbers may efficiently compete with early mRNAs at the ribosome level if some component of the translational system is limiting in the cell. Alternatively, late mRNA may specify a product inhibitory for the expression of at least some early mRNA. This would explain both the absence of certain early products, like E 19,000-dalton and E 11,000-dalton polypeptides, from the *in vitro* translation products (Fig. 1, lane 2) as well as the apparent lack of helper functions for AAV2 growth in cells injected with late Ad2 mRNA or a mixture of early and late Ad2 mRNAs (Fig. 4, lanes 5 and 6).

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