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A Cascade of Adenovirus Early Functions Is Required for Expression of Adeno-Associated Virus

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Summary

One measurable biological activity of early adenovirus genes is their ability to promote growth of the defective adeno-associated virus, AAV. We have identified an ordered sequence of communications among the early genes of adenovirus type 2 (Ad2) that results in expression of the helper activity. We purified DNA fragments and mRNAs corresponding to early Ad2 regions E1, E2A, E3 and E4 and injected them via glass capillaries into AAV-infected cells. DNAs were placed in the nucleus, mRNAs in the cytoplasm. AAV DNA and proteins synthesized in response to the injected Ad2 nucleic acids were extracted from as few as 100 cells and identified by gel electrophoresis. Our results reveal a cascade of early Ad2 gene regulation, E1 → E2A → E4, with E4 providing the helper effect for AAV.

Introduction

The early adenovirus genes are arranged in overlapping clusters at several well-separated locations on both strands of the viral DNA. Each early region gives rise to a number of mRNAs specifying polypeptides of largely unknown function that mediate the beginning of the virus growth cycle (for review, see Ziff, 1980). Figure 1 shows a diagram of the adenovirus type 2 (Ad2) genome with map positions of the early mRNAs. The bulk of these mRNAs comes from the major gene regions E1A, E1B, E2A, E3 and E4. In addition, a number of rare species of early RNA have recently been mapped in the area marked E2B and in the region of the major late promoter (Chow et al., 1979; Galos et al., 1979; Kitchingman and Westphal, 1980; Stillman et al., 1981).

The sequential appearance of early mRNAs (Nevins et al., 1979) and proteins (Neuwald et al., 1977) suggests that the early genes interact to control their own expression. Preliminary evidence for regulatory communication among the individual gene clusters comes from experiments dealing with the effect of mutations in defined early genes on the overall pattern of early gene expression. Mutations in E1A, a region expressed very early, cause a general shut-off of early RNA synthesis, suggesting that expression of E1A is required to turn on distant early genes (Berk et al., 1979; Jones and Shenk, 1979). Similarly, a protein product of region E2A appears to modulate transcription from other early gene regions (Carter and Blanton, 1978; Nevins and Winkler, 1980).

The complex interactions among the early adenovirus genes render it difficult to distinguish direct from indirect or regulatory effects of early mutations. We have attempted to circumvent this problem by microinjecting subsets of Ad2 genes and products into permissive cells and measuring their biological activities in this simplified system. The effect we chose to analyze was the ability of adenovirus to promote the growth of AAV. AAV is a defective parvovirus unconditionally dependent on a coinfecting adenovirus for its own replication (for review see Ward and Tattersall, 1978). In the absence of a helper virus, no AAV macromolecular synthesis can be detected. In a double infection with adenovirus, synthesis of AAV DNA coincides with that of adenovirus DNA, and synthesis of AAV RNA coincides with late RNA (Carter et al., 1973), suggesting that AAV expression is contingent on factors also implicated in the adenovirus late switch. Early adenovirus gene expression is necessary and sufficient to provide the helper effect, because cells infected with AAV and injected with early Ad2 RNA produce infectious AAV (Richardson et al., 1980). Recent studies with mutants of Ad2 and Ad5 have implicated early regions E1 and E2A in the helper effect (Myers et al., 1980; Ostrove and Berns, 1980; Janik et al., 1981; Jay et al., 1981; but see Straus et al., 1976a). Janik et al. (1981), who transfected purified adenovirus DNA fragments into AAV-infected cells, found that region E4 is also involved in the process. The biochemical basis of AAV defectiveness is unknown, although studies with an Ad5 mutant (Myers and Carter, 1981; Jay et al., 1981) suggest that AAV growth may be blocked at more than one stage in its life cycle.

While a role in the helper effect was thus established for several early gene regions of adenovirus, the cited experiments did not comment on the nature of that role. The purpose of our study was to distinguish the Ad2 genes directly involved in helper activity from those, if any, that control their expression. To this end, we purified DNA fragments and mRNAs corresponding to regions E1, E2A, E3 and E4 of Ad2 and injected them into AAV-infected cells. After the helper effect had been expressed, AAV DNA or proteins were extracted from the injected cells and identified by gel electrophoresis.

Results

Early mRNAs Selected by Hybridization to Ad2 DNA Fragments Express Characteristic Polypeptides in Vitro

We prepared bulk RNA from HeLa cells infected with Ad2 and grown for 9 hr in the presence of cycloheximide. The drug prevents the switch to late viral

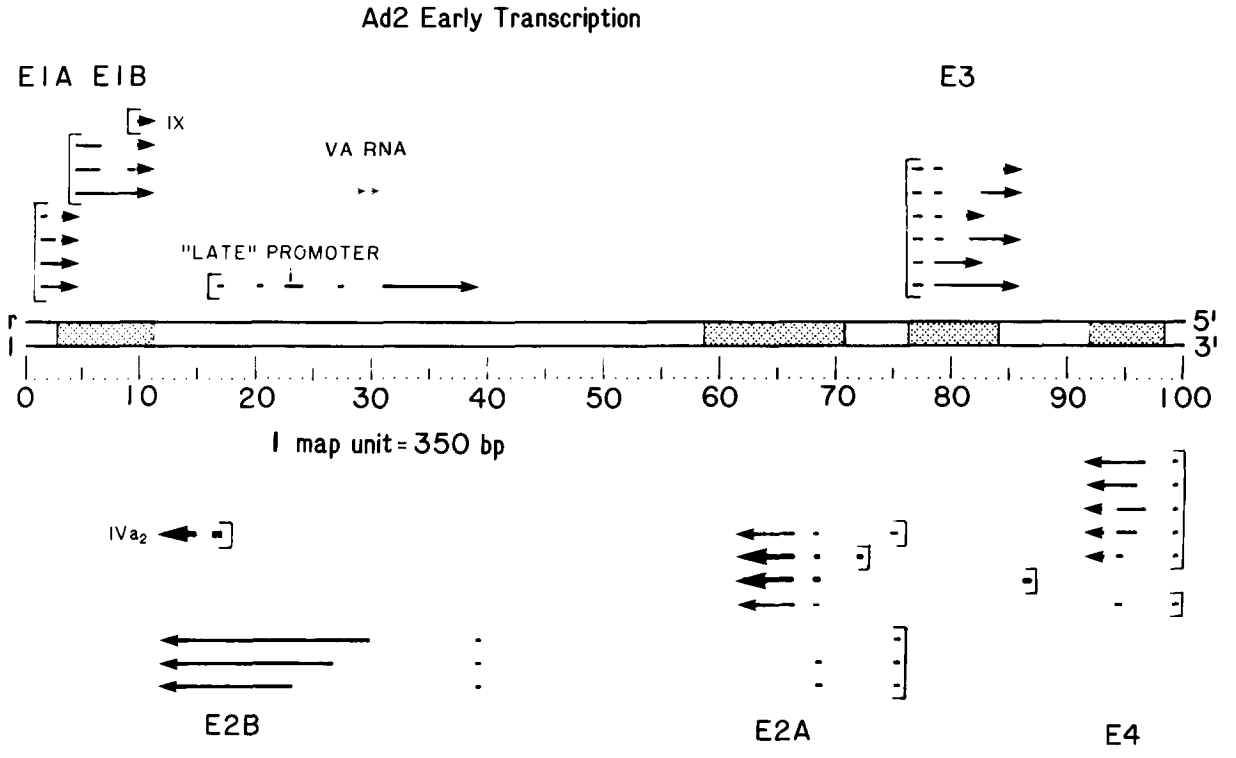


Figure 1. Map of the Ad2 Genome and the Early Transcripts

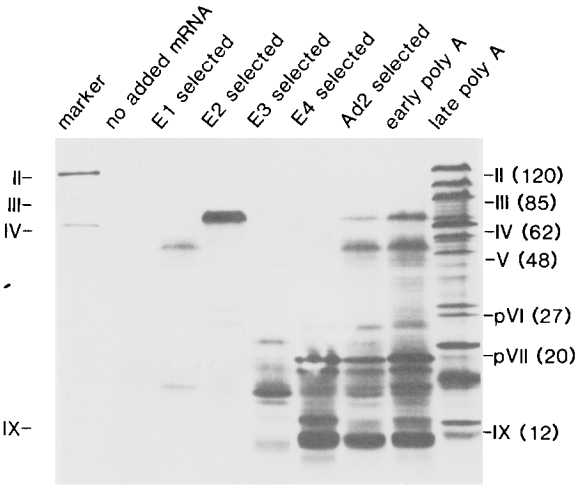
The figure represents data from several laboratories (reviewed by Ziff, 1980) and has been adapted from Chow et al. (1980). Numbering of the early gene regions follows the nomenclature of Kitchingman et al. (1977) and Stillman et al. (1981). Brackets: promoter locations. Most RNAs are spliced, hence the interruption of lines. Arrows: direction of transcription. RNAs mapping outside the indicated regions include rare transcripts starting at the late promoter and containing 'i" sequences, as well as RNAs that begin to accumulate early but specify late products, such as proteins IVa_2 and IX. The VA RNAs are small polymerase III transcripts made throughout the virus life cycle. Stippled areas: DNA fragments used in this study for selection of early mRNAs. They map at positions 2.8 to 11.1 (Sma I E), 58.5 to 70.7 (Eco RI B), 75.9 to 84.0 (Eco RI D) and 91.9 to 98.3 (Sma I G). These and other restriction sites quoted in this paper were obtained from Tooze (1980).

expression and increases the accumulation of early RNA (Parsons and Green, 1971; Nevins et al., 1979). Poly(A)-containing RNA was selected by oligo(dT)-cellulose chromatography and fractionated by hybridization to cloned Ad2 DNA sequences corresponding to regions E1, E2A, E3 and E4 (Figure 1, hatched areas).

The purity and integrity of the purified mRNAs was tested by cell-free translation. The translation products (Figure 2) compare well with those described in the literature. E1 RNA specified a polypeptide of 15.5 kilodaltons (15.5 kd) and several others in the range of 45 to 55 kd. By comparison, Halbert et al. (1979) found translation products of 35, 41, 47 and 53 kd from E1A, and 15 and 52 kd from E1B. The major product of E2A mRNA corresponds to the E2A/72 kd polypeptide ascribed to this region (Lewis et al., 1976). Note that no trace of the E2A/72 kd band is seen in the neighboring lanes, attesting to the accuracy of the purification procedure. The two minor bands of 25 and 28 kd were not noticed by others, and their relationship to the E2A/72 kd polypeptide remains to be established. E3 RNA gave rise to several polypeptides between 20 and 10 kd, with prominent bands at 20, 14.5, 13.5 and 10 kd. Persson et al. (1980) described three cell-free translation products, E3/16, E3/14.5 and E3/14 kd and identified the E3/16 kd polypeptide as the precursor to the 19 kd glycoprotein of Ad2. Possibly our cell-free system is able to process the 16 kd precursor to the mature form. This might explain the E3/20 kd band that we observe. In our experiments, E4 RNA specified many (>10) polypeptides in the range of 25 to 8 kd, with major bands at 18.5, 17, 12.5, and 11 kd. Other laboratories have reported five to seven E4 translation products in the size range seen here (Harter and Lewis, 1978; Lewis and Mathews, 1980; Persson et al., 1980). We used a large DNA excess for our hybridization selections, and that may be required to accumulate mRNA species encoding minor polypeptides.

The Helper Effect for AAV Is Encoded in the E4 Region of Ad2

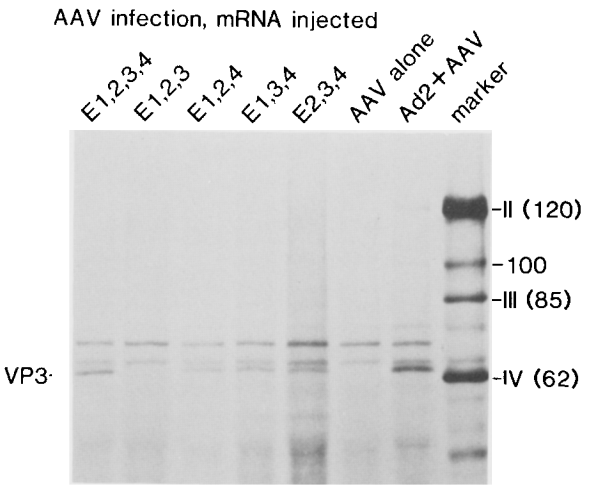
Vero, the cell line chosen for our injection experiments, is fully permissive for AAV growth in the presence of an Ad2 helper (Richardson et al., 1980). We injected the selected early Ad2 mRNAs via glass cap-



cell-free translation of purified mRNAs

Figure 2. Polypeptides Derived from Cell-Free Translation of Ad2 mRNAs

Proteins were separated by electrophoresis through a 17% polyacrylamide gel. Individual 15 μl assays contained 0.5 μg of early or late poly(A)-containing RNA (rightmost two lanes), or 0.5 μl of RNA selected by hybridization to Ad2 virion DNA or cloned restriction fragments (Figure 1) as described in Experimental Procedures. In this and subsequent figures, unless otherwise stated,the marker lane represents total cell proteins labeled in vivo with ^{35}S-methionine late after Ad2 infection. Individual late proteins are identified in the margins, following the nomenclature of Lewis et al. (1974). Approximate molecular weights in kd are in parentheses.



immunoprecipitates with anti-AAV serum

Figure 3. Polypeptides Precipitated with Anti-AAV2 Serum

Cells were infected with AAV2 and either injected with purified Ad2 mRNAs or coinfected with Ad2, as indicated. Proteins were separated on a 13% polyacrylamide gel and visualized by fluorography (Bonner and Laskey, 1974). All immunoprecipitates in this and subsequent figures were produced from 100 to 200 cells. Cells injected with RNA were infected with AAV one hour later and labeled with ^{35}S-methionine from 5–15 hours after infection. The anti-AAV2 serum used precipitates three AAV2 capsid proteins designated VP1, VP2 and VP3 with molecular weights of approximately 97, 77 and 63 kd, respectively. VP3 is the major component and constitutes 85% of the protein mass of the virion (Rose et al., 1971). In this figure, only VP3 is visible and is indicated in the left margin. Only those cells that did not receive E4 RNA failed to produce a detectable VP3 band.

illaries (Graessmann et al., 1980) into the cell cytoplasm. Within one hour, the cells were infected with AAV2, and from 5 to 15 hr after infection they were incubated in the presence of ^{35}S-methionine. The labeled cells were lysed, and polypeptides immunoprecipitated with anti-AAV serum were separated by polyacrylamide gel electrophoresis. In preliminary tests (not shown) we demonstrated that both total early RNA selected by hybridization to Ad2 DNA and a mixture of E1 + E2A + E3 + E4 mRNAs promoted AAV antigen expression. We therefore combined and injected the four selected mRNAs in groups of three, omitting one at a time (Figure 3). Only that combination lacking E4 mRNA failed to support AAV expression. In a complementary experiment, each selected RNA was injected singly. As seen in Figure 4, only cells that received E4 mRNA expressed the AAV polypeptides.

In a regular infection, AAV gene expression is preceded by and dependent on AAV DNA replication (Carter et al., 1973). The experiment whose results are shown in Figure 5 demonstrates that AAV DNA replication also takes place in Vero cells injected with E4 mRNA and infected with AAV. Total cell DNA was extracted two days after infection and digested with restriction nuclease Bal I. The enzyme cuts AAV DNA near both ends (Lusby et al., 1980) and generates one major fragment of 0.95 unit length, from monomeric as well as multimeric (Straus et al., 1976b) forms of the genome. After agar gel electrophoresis and blotting (Southern, 1975), AAV DNA was hybridized with nick-translated (Rigby et al., 1977) AAV ^{32}P-DNA probe and visualized by autoradiography. Other lanes of the gel contained known amounts of AAV DNA cut with Bal I. Aware of the quantitative limitations of the blotting technique, we estimate that a double infection with Ad2 helper virus, included as a control, yielded roughly 5×10^6 copies of full-size AAV DNA per cell. Injection of Ad2 DNA produced about 5×10^4 copies and injection of E4 mRNA about 5×10^3 copies of full-size AAV DNA per cell. Cells infected with AAV alone were found to contain <200 DNA copies. In addition to full-length molecules, a considerable quantity of smaller AAV DNA fragments were detected, mainly in the cells injected with E4 mRNA, but also in the controls. These smaller DNAs probably represent defective genomes with internal deletions, routinely found in AAV-infected cells (Carter et al., 1979; Hauswirth and Berns, 1979). The significance of the increased relative abundance of defective genomes in cells injected with E4 RNA is not known. We concluded, however, that the helper effect exerted by E4 mRNA extended to both AAV DNA replication and protein synthesis.

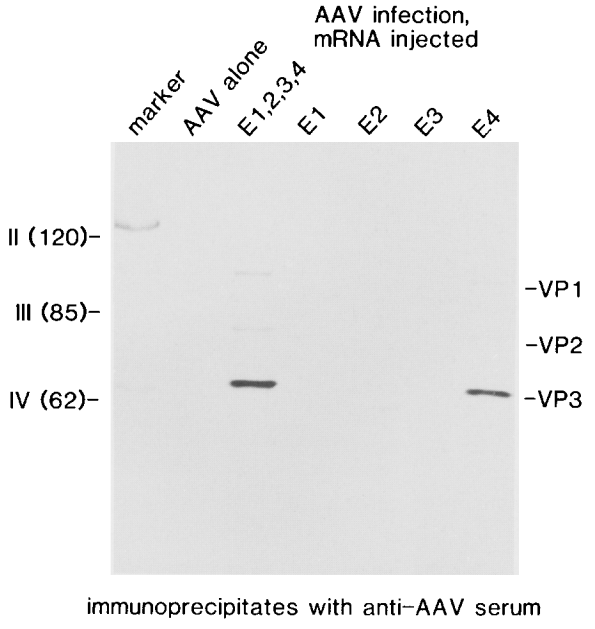


Figure 4. Polypeptides Precipitated with Anti-AAV2 Serum

Cells were infected with AAV2 and injected with purified Ad2 mRNAs. Experimental design is outlined in the legend to Figure 3. Cells injected with a mixture of E1, E2A, E3 and E4 RNAs, or with E4 RNA alone, produced AAV2 capsid proteins, indicated in the right margin.

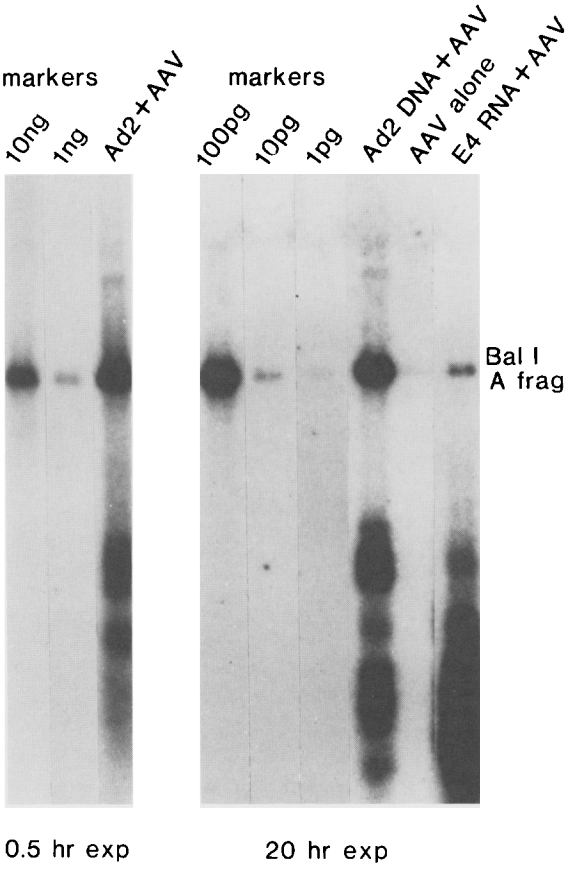


Figure 5. Southern Blot Analysis of AAV DNA Sequences

Cells were infected with AAV and injected with either E4 RNA or Ad2 virion DNA (0.5 mg/ml), or coinfected with Ad2. Cells injected with DNA or RNA were infected with AAV one hour later and harvested at 48 hr after infection. DNA was prepared from 500 injected cells, or 1000 doubly infected cells. Marker lanes contain indicated amounts of AAV2 double-stranded DNA digested with Bal I. All samples were subjected to electrophoresis, transferred and hybridized simultaneously, and this figure is a composite of two different exposures of the same blot. The position of the AAV2 Bal I A fragment (95% full length) is indicated in the margin. The heterogeneous shorter fragments are probably products of aberrant replication (see Results). AAV DNA clearly replicated in cells injected with E4 RNA (extreme right lane).

Expression of the E4-Coded Helper Effect Is Mediated by a Regulatory Cascade, E1 → E2A → E4

Our finding that E4 mRNA was necessary and sufficient to provide the helper effect for AAV had to be reconciled with the work of other laboratories (see Introduction), which ascribe a role in this process to early regions E1, E2A and E4. One way to accommodate all these findings was to postulate that E1 and E2A genes participate in a regulatory pathway leading to E4 expression. It was possible to test this hypothesis by injecting various combinations of DNA fragments and mRNAs corresponding to the Ad2 early regions into AAV-infected cells. While RNA was routinely injected into the cytoplasm, our first tests with DNA fragments (not shown) confirmed the experience of Capecchi (1980), who noted that efficient expression of injected DNA requires placement into the nucleus. We purified suitable fragments spanning all of region E1, E2A and E4 from restriction digests of Ad2 DNA. These fragments were injected, singly and in combination, into Vero cell nuclei. After AAV infection and a 16 hr incubation period, the cells were pulse-labeled with ^{35}S-methionine and analyzed for AAV antigen expression as before. The results of this experiment are shown in Figure 6. Neither E4 DNA nor any other fragment by itself was able to promote AAV expression. Only E1, E2A and E4 DNAs injected together provided the helper effect. This was the result to be expected if E1 and E2A genes mediate E4 gene expression.

Gene products, rather than the genes themselves, are the likely regulatory elements in early adenovirus gene control. Therefore, since E4 DNA is expressed when injected together with E1 and E2A DNA, one would expect the same result if E1 and E2A mRNAs plus E4 DNA were injected. Figure 7 shows that this is indeed the case. In this experiment, RNAs were injected into the cytoplasm, DNA into the nucleus. After AAV infection and an appropriate incubation period, cells were labeled and examined for the presence of AAV antigens. Not only did the cells injected with E4 DNA and both E1 and E2A mRNAs express the helper effect, but so did the cells that received E4 DNA and E2A mRNA alone. This result implies that

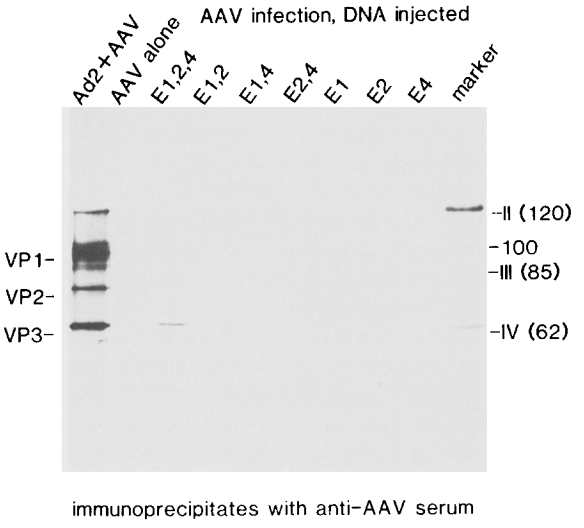


Figure 6. Polypeptides Precipitated with Anti-AAV2 Serum

Cells were infected with AAV2 and injected with purified restriction fragments of Ad2. Fragments chosen were Bam HI B (E1 DNA, 0 to 29.0 map units), Sma I A (E2A DNA, 56.9 to 75.8) and Eco RI C (E4 DNA, 89.7 to 100). Fragments were injected either singly or combined into cell nuclei at concentrations equimolar to a 0.5 mg/ml solution of Ad2 DNA. One hour later the cells were infected with AAV2, and about 16 hr after infection were labeled for 2 hr with ^{35}S-methionine. Only those cells injected with all three DNA fragments responded by synthesizing AAV2 antigen.

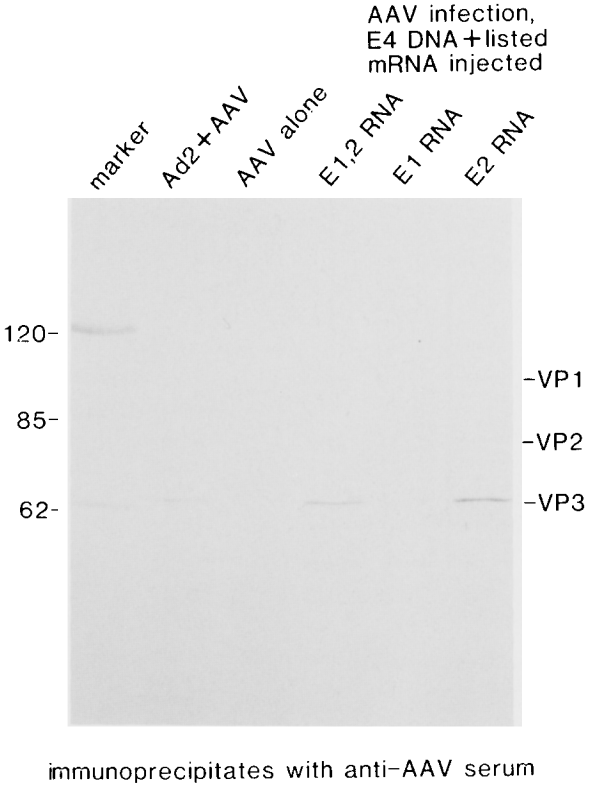


Figure 7. Polypeptides Precipitated with Anti-AAV2 Serum

Cells were infected with AAV2 and injected with E4 DNA plus purified Ad2 mRNAs. Cells were injected with the purified mRNAs indicated in the figure, and incubated at 37°C. One hour later the same cells were injected with E4 DNA (see Figure 6). After another hour at 37°C the cells were infected with AAV2 and labeled from 5 to 15 hr after infection with ^{35}S-methionine. Only cells receiving both E2A RNA plus E4 DNA produced AAV capsid proteins.

E2A gene expression is necessary and sufficient to elicit the E4-encoded helper effect.

The fact that injection of E1 mRNAs and E4 DNA did not promote AAV expression indicated that the role of E1 gene functions in the helper effect did not involve a direct interaction with E4 DNA. Instead, it appeared likely that E1 products acted to promote E2A expression, which in turn induced the E4-encoded helper effect. To test this idea, we injected E1 mRNA into the cytoplasm and E2A plus E4 DNAs into the nucleus, infected the cells with AAV and monitored AAV antigen synthesis. As shown in Figure 8, cells treated as described produced AAV capsid proteins, whereas cells injected with either the RNA or the DNAs alone did not. Taken together, these experiments imply that the Ad2 helper effect for AAV is established as a result of a regulatory pathway, involving the expression of a number of genes in the ordered sequence E1 → E2A → E4 → AAV.

Discussion

Our results show that the helper effect for AAV resides in early region 4, located at the right end of the adenovirus gene map. The helper effect is trans-acting and presumably provided by one or more proteins encoded in the E4 gene cluster. Several distinct mRNA species (Figure 1) and polypeptides (Figure 2) map in the E4 region, and each of them is at present a candidate for the helper activity. A more refined definition may become possible once fractionated E4 mRNA species are available for microinjection. To the best of our knowledge, the helper effect for AAV is the first biological activity shown to reside in the E4 region of adenovirus. As mutants in E4 are now being isolated (Challberg and Ketner, 1981), more information concerning E4 functions will, we hope, emerge.

We find that both AAV replication and protein synthesis are turned on by the helper effect. Since AAV DNA synthesis precedes gene expression (Carter et al., 1973), we may assume that AAV replication, transcription, protein synthesis and assembly is the sequence of events triggered by E4. The helper effect may come in at one or more steps along the way, and it may be mediated by one or several E4 products. Physical characterization of these products will be a necessary first step toward analyzing their mode of action.

The discovery of a regulatory cascade, E1 → E2A → E4, emphasizes the intricacy of adenovirus gene regulation. While all of these early regions had already been implicated in the helper effect for AAV (see

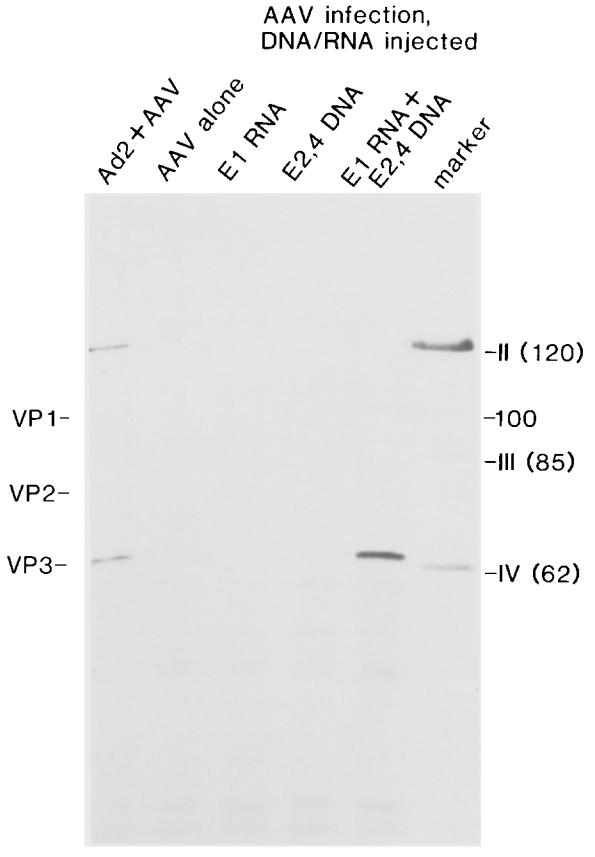


Figure 8. Polypeptides Precipitated with Anti-AAV2 Serum

Cells were infected with AAV2 and injected with Ad2 DNA fragments and purified mRNA, as indicated. Cells receiving either E1 RNA alone, or a mixture of E2A DNA and E4 DNA, were treated as described in Figure 3. Cells receiving both DNA and RNA were treated according to the protocol described in Figure 7. Only the cells receiving both E1 RNA and the mixture of E2A DNA and E4 DNA produced AAV capsid proteins.

Introduction), the role each of them plays in the pathway of controls can only now be assessed. E1A had been recognized before as the site of functions required for expression of other early regions (Berk et al., 1979; Jones and Shenk, 1979). Although our experiments do not distinguish between E1A and E1B, we speculate that it is E1A that plays the pivotal role in generating the helper effect for AAV.

More of a problem is encountered when considering the role of E2A in the regulatory cascade. This region is the site of the 72 kd DNA-binding protein (DBP), a polypeptide with several functions. Apart from its direct involvement in DNA replication, DBP regulates its own expression and influences the adenovirus host range (for review, see Ziff, 1980). Our finding that E2A mRNA is needed to turn on the E4-encoded helper effect seems to offer yet another role for DBP as a positive regulator of E4 transcription. That interpretation, however, cannot easily be reconciled with a number of published observations. First, Nevins et al. (1979) noted that E4 mRNA synthesis proceeds at maximal rates several hours before that of E2A. However, low levels of E2A transcripts can be found in the cytoplasm as early as 2 hr after infection (Neuwald et al., 1977). Second, Nevins and Winkler (1980), studying the effect of a mutation in the gene for DBP on overall early gene expression, concluded from their experiments that E2A exerts a negative control on E4 transcription. Results of an earlier study of the same mutation (Carter and Blanton, 1978) were also consistent with this interpretation. If we wish to maintain that E2A acts at the level of E4 transcription when mediating the helper effect, we would have to argue that DBP or an as-yet-unknown E2A gene product accumulates early enough to turn on a minor species of E4 mRNA. This RNA would then specify the helper effect, while the bulk of E4 transcription would be controlled differently. An attractive alternative to this line of reasoning is to assume that E2A exerts positive control over E4 at a post-transcriptional level. Klessig and Chow (1980), working with an adenovirus with altered host range caused by a mutation in the DBP gene, provided data suggesting that DBP has a function in RNA processing. In addition, Jay et al. (1981) observed an apparent translational block of AAV mRNA in cells coinfected with H5*ts*125, another mutant in the DBP gene. Whatever controls the E2A gene product exerts to activate the E4-coded helper effect, whether transcriptional or post-transcriptional, they are clearly bypassed by injecting purified E4 mRNA into the cell cytoplasm.

The cascade E1 → E2A → E4 should be considered a minimal requirement for AAV expression under our test conditions. Additional factors may be at work in tissue culture cells or in the living organism to generate infectious AAV. For instance, Janik et al. (1981) noticed a requirement of the gene for one of the Ad2 virus-associated RNAs, VA1 (for review, see Ziff, 1980). These investigators measured AAV growth in cells transfected with adenovirus DNA fragments and noted that AAV virions were no longer produced when the VA1 gene was cut by the restriction endonuclease Bam HI. We performed a similar experiment in our microinjection assay and did not detect any requirement for the VA1 gene (not shown). This may mean that an intact VA1 gene is of importance at a late step of AAV expression (for example, during virus assembly) and brings to mind experiments by Mayor et al. (1977), who noted that AAV production is low in cells coinfected with an adenovirus mutant defective in assembly. Alternatively, VA1 may boost the efficiency of one or more steps in the regulatory cascade. We could conceivably overlook such an effect because we inject many (~100) gene copies per cell. It is probable that a closer look at the regulation of the

AAV helper effect, which is just one of many early adenovirus gene functions, will reveal even more ramifications than the experiments reported here indicate.

Experimental Procedures

Cells and Viruses

The Vero line of African Green monkey kidney cells was propagated and prepared for microinjection as described by Richardson et al. (1980). For purification of Ad2 stocks, see Meyer et al. (1977). AAV2 stocks (provided by B. Carter) were grown in KB cells coinfected with Ad2. Cells were frozen and thawed three times, and the helper virus was inactivated at 60°C for 20 min. AAV titers were determined by a fluorescent focus assay (Myers et al., 1980).

Purification of Early Ad2 mRNAs

HeLa spinner cells were infected with 10^4 Ad2 particles per cell and grown for 9 hr in the presence of 25 μg/ml cycloheximide. ^{3}H-uridine (10 μCi/ml) was added during the final 2 hr. Cytoplasmic extract was prepared by homogenization in reticulocyte standard buffer (Penman et al., 1969), and made 0.5% (w/v) SDS. Thereafter, the preparation of poly(A)-containing RNA was as described by Eron et al. (1974). Ad2 mRNAs were selected by hybridization of total RNA to cloned restriction fragments of Ad2 DNA. All recombinant plasmids containing Ad2 DNA were made in our laboratory by G. R. Kitchingman. Sma I fragments E (region E1) and G (region E4) were inserted into the Eco RI site of plasmid pBR325 (Bolivar, 1978), with use of Eco RI linkers. Eco RI fragments B (region E2A) and D (region E3) were inserted into plasmid pBR322 (Bolivar et al., 1977). The following steps followed essentially the methods cited or described by Parnes et al. (1981). DNA was purified from plasmids grown in E. coli strain LE 392 and immobilized on nitrocellulose filters (five filters 24 mm diameter each containing ~40 μg for each of the four probes). The hybridization mixture (2 ml) contained 400 μg of total early poly(A)-containing RNA and the 20 filters carrying the equivalent of about 1 mg of Ad2 DNA. After washing, the four batches of filters were sorted into siliconized glass vials and specific RNA was eluted for 2 min at 100°C in 1 ml of sterile H_2O containing 5 μg of carrier tRNA. The eluate was frozen in a dry ice/ethanol bath, thawed and stored on ice. The elution procedure was repeated and the RNA contained in the combined eluates was separated from contaminating DNA by oligo(dT) column chromatography (100 μl bed volume). Each batch of RNA was stored under liquid nitrogen in 5 μl of 10 mM Tris–HCl (pH 7.5). Activity and purity of the selected mRNAs was checked by cell-free translation, as described by Richardson et al. (1980). The extent of cross-contamination was very small to undetectable, judging by the translation products (see Figure 2). Occasionally E1, E2A and E3 RNAs contained small amounts of E4 RNA, although E4 RNA itself always appeared free of contamination. Contamination by non-polyadenylated RNAs, although not assessed directly, is unlikely, considering the stringency of the purification procedure.

Preparation of Ad2 DNA Restriction Fragments

Ad2 DNA fragments to be used for microinjection (see Figure 6) were prepared by cleaving virion DNA (Pettersson and Sambrook, 1973) with the appropriate restriction endonuclease. Sma I A was prepared from a Sma I/Xba I double digest to avoid contamination with Sma I B (18.5 to 36.7). After agarose gel electrophoresis and elution, fragments were concentrated by DE-23 anion exchange chromatography, extracted three times with phenol, once with chloroform and finally stored in sterile 10 mM Tris–HCl (pH 7.5) at concentrations equimolar to a 2 mg/ml solution of Ad2 DNA. DNA fragments prepared in this way were judged to be free of neighboring fragments by agarose gel electrophoresis under conditions that would reveal less than 2% molar contamination.

Microinjection

Our application of the technique of Graessman et al. (1980) has been described by Richardson et al. (1980). Injection capillaries were filled from the mouth (0.5 mm diameter) of a siliconized glass micropipet containing the amount of nucleic acid (about 0.2 μl) sufficient for one experiment. Based on certain assumptions stated in Richardson et al. (1980), we estimate the number of RNA molecules injected into each cell to be a few thousand, and the number of copies of each DNA fragment to be approximately 100.

Analysis of AAV Expression

Methods for polypeptide analysis have been described by Richardson et al. (1980) with the exceptions that cells were labeled with ^{35}S-methionine only, and cell lysates were cleared with normal rabbit serum prior to immunoprecipitation with anti-AAV serum. The latter serum was made in rabbits by injection of intact AAV virions that had been banded three times in CsCl. It is not known whether it precipitates the three virion polypeptides VP1, VP2 and VP3 independently, or only in association as intact virions.

Cells to be examined for AAV DNA synthesis were lysed in 40 μl of 0.5% SDS, 5 mM EDTA and 10 mM Tris–HCl (pH 7.0) contained around the cell island as described by Richardson et al. (1980). Carrier tRNA (5 μg) was added, and the lysate was digested with 0.2 mg/ml pronase for 10 min at 37°C. DNA was extracted with phenol, fragmented with Bal I enzyme in the presence of RNAase A, subjected to agarose gel electrophoresis with salmon sperm DNA present as carrier, transferred to nitrocellulose paper (Southern, 1975) and hybridized (Wahl et al., 1979) with nick-translated (Rigby et al., 1977) AAV ^{32}P-DNA (2×10^8 cpm/μg).

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