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

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(Ad2)

[redacted]

identified an ordered sequence of communications [redacted] **that results in expression of the helper activity. We** [redacted] **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** [redacted]

Introduction

The early adenovirus genes are arranged in overlap- [redacted] rise to a number of mRNAs specifying polypeptides of [redacted] 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. [redacted] region of the major late promoter (Chow et al., 1979; [redacted]

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 [redacted] 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 [redacted] jecting subsets of Ad2 genes and products into permissive cells and measuring their biological activities [redacted] 1978). In the absence of a helper virus, no AAV [redacted] ble infection with adenovirus, synthesis of AAV DNA [redacted] switch. Early adenovirus gene expression is necessary and sufficient to provide the helper effect, be- [redacted] 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 [redacted] is unknown, although studies with an Ad5 mutant [redacted] 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 [redacted] those, if any, that control their expression. To this [redacted] 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.

[redacted]

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

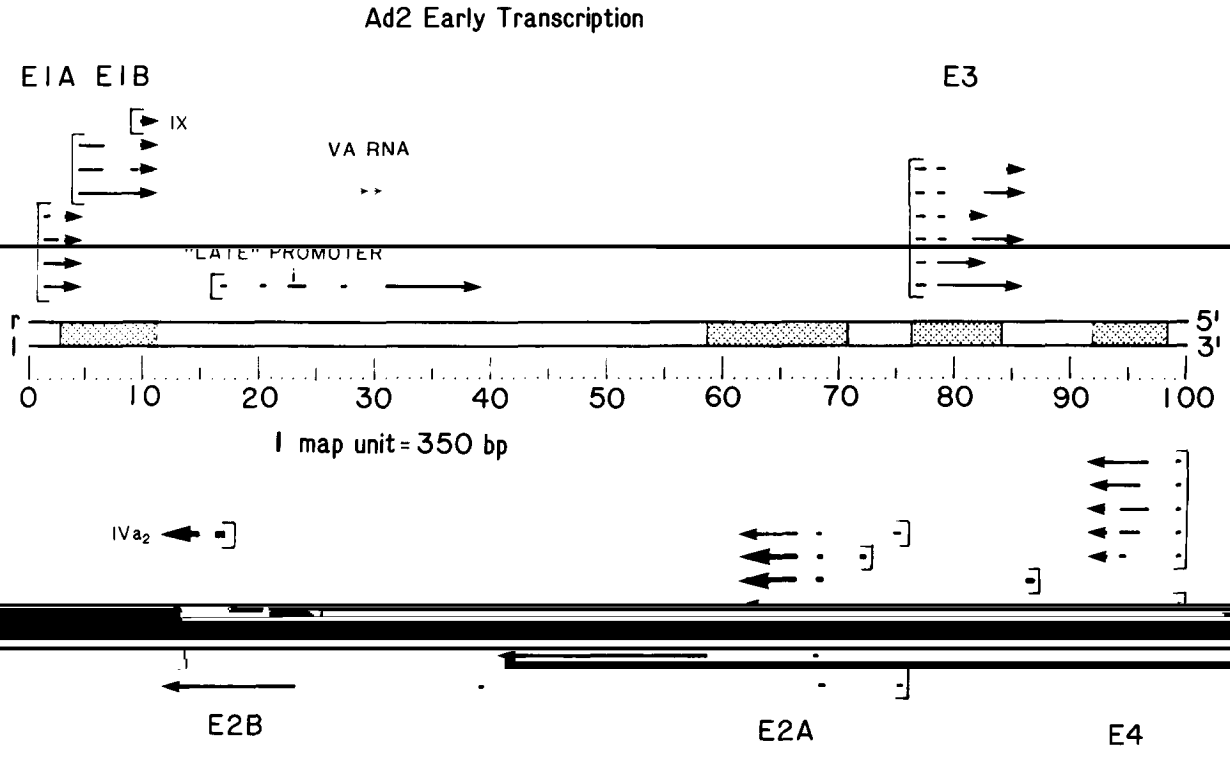


Figure 1. Map of the Ad2 Genome and the Early Transcripts

early gene regions follows the nomenclature of Kitchingman et al. (1977) and Stillman et al. (1981). Brackets: promoter locations. Most RNAs are

proteins IVa_2 and IX. The VA RNAs are small polymerase III transcripts made throughout the virus life cycle. Stippled areas: DNA fragments used

expression and increases the accumulation of early RNA (Parsons and Green, 1971; Nevins et al., 1979).

cellulose chromatography and fractionated by hybridization to cloned Ad2 DNA sequences corresponding

areas).

The purity and integrity of the purified mRNAs was

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

bands of 25 and 28 kd were not noticed by others, and their relationship to the E2A/72 kd polypeptide

bands at 20, 14.5, 13.5 and 10 kd. Persson et al. (1980) described three cell-free translation products,

16 kd polypeptide as the precursor to the 19 kd glycoprotein of Ad2. Possibly our cell-free system is

form. This might explain the E3/20 kd band that we observe. In our experiments, E4 RNA specified many

al., 1980). We used a large DNA excess for our hybridization selections, and that may be required to accumulate mRNA species encoding minor polypeptides.

Vero, the cell line chosen for our injection experiments, is fully permissive for AAV growth in the pres-

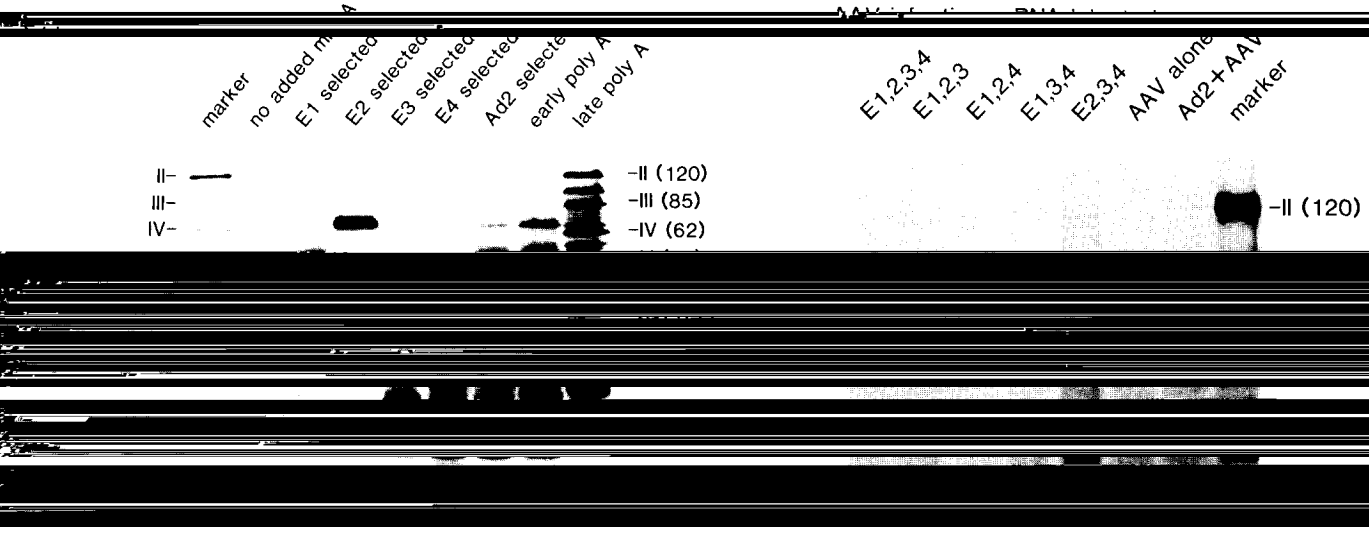


Figure 2. Polypeptides Derived from Cell-Free Translation of Ad2 mRNAs

Proteins were separated by electrophoresis through a 7% polyacryl-

lected by hybridization to Ad2 virion DNA or cloned restriction frag-

represents total cell proteins labeled in vivo with ^{35}S-methionine late after Ad2 infection. Individual late proteins are identified in the mar-

molecular weights in kd are in parentheses.

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

figures were produced from 100 to 200 cells. Cells injected with RNA

tates three AAV2 capsid proteins designated VP1, VP2 and VP3 with

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 cyto-

incubated in the presence of ^{35}S-methionine. The

precipitated with anti-AAV2 serum were separated by

forms of the genome. After agarose gel electrophoresis,

ized with nick-translated (Rigby et al., 1977) AAV ^{32}P-

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 2). Only that combina-

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 $5 \times$

tion is shown. In a complementary experiment, each selected RNA was injected singly. As seen in Figure 4, only cells that received E4 mRNA suppressed the AAV poly-

copies of full-size AAV DNA per cell. Cells infected with AAV alone were found to contain <200 DNA

peptides.

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

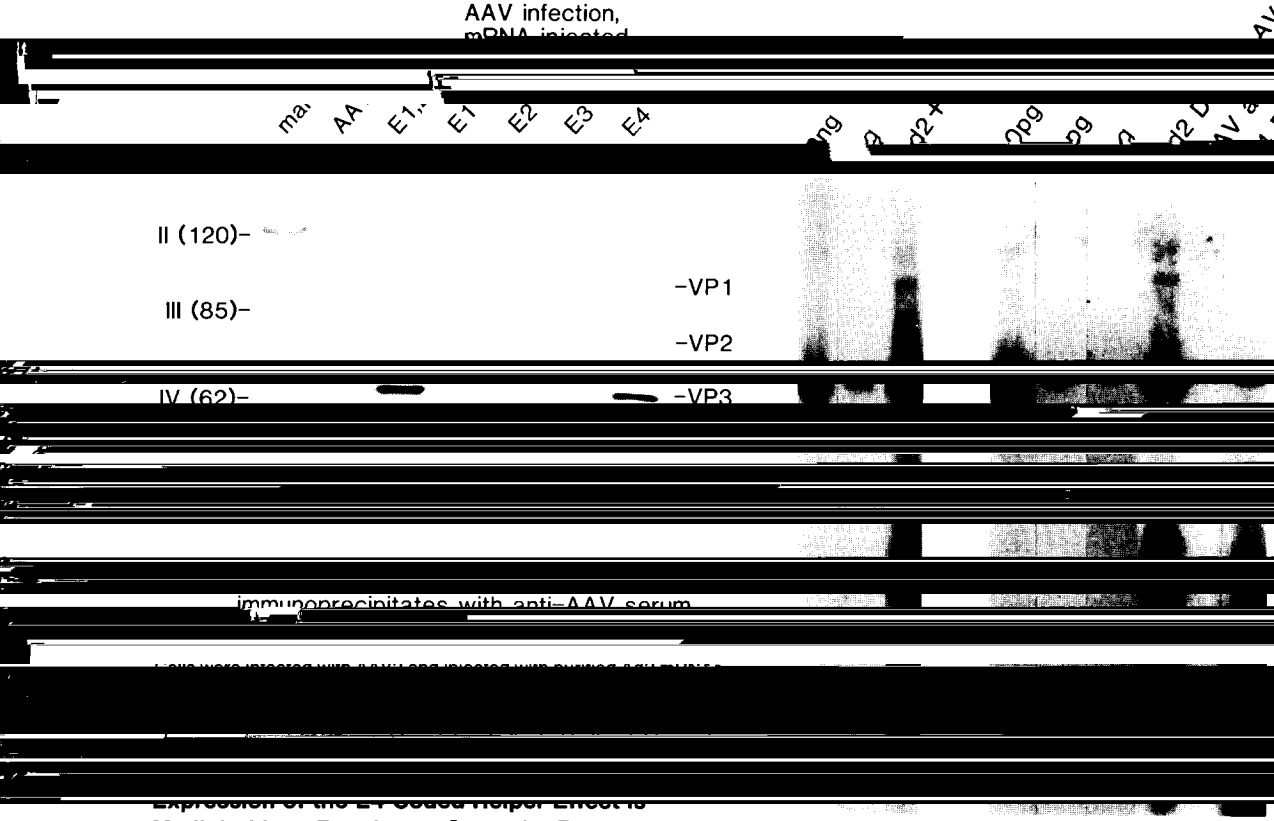
erable quantity of smaller AAV DNA fragments were detected, mainly in the cells injected with E4 mRNA, but also in the controls. These smaller DNAs probably represent defective genomes with internal deletions, routinely found in AAV-infected cells (Carter et al., 1979; Hauswirth and Berns, 1979). The significance

extracted two days after infection and digested with

nomes in cells injected with E4 RNA is not known. We

near both ends (Lusby et al., 1980) and generates one major fragment of 0.95 unit length, from mono-

E4 mRNA extended to both AAV DNA replication and protein synthesis.



immunoprecipitates with anti-AAV serum

Figure 5. Southern Blot Analysis of AAV DNA Sequences

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

of AAV2 double-stranded DNA digested with Bal I. All samples were subjected to electrophoresis, transferred and hybridized simultane-

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

modate 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. Gene products, rather than the genes themselves,

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-

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

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

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

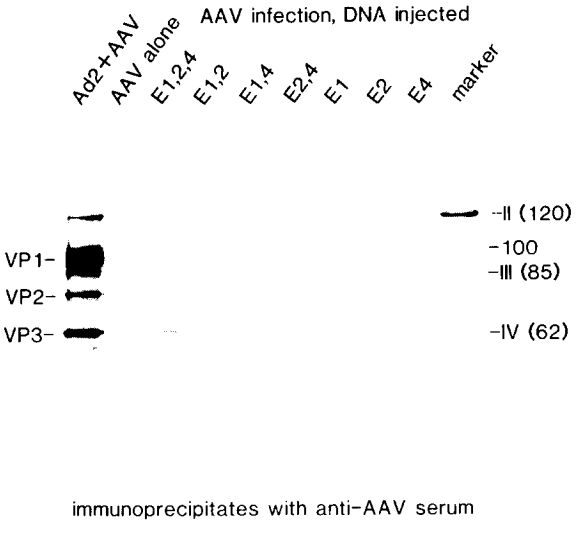


Figure 6. Polypeptides Precipitated with Anti-AAV2 Serum

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, 80.7 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 Only those cells injected with all three DNA fragments responded by

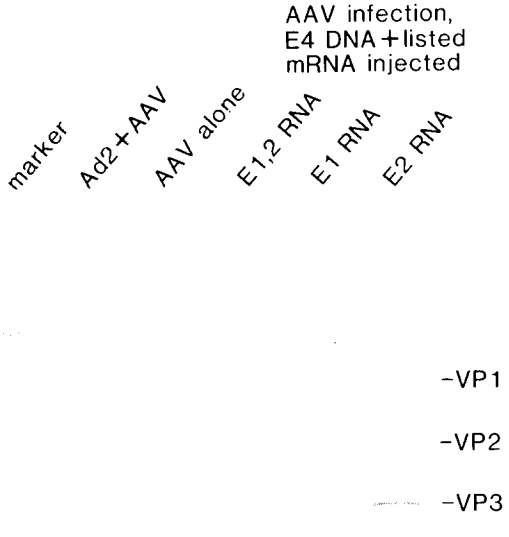


Figure 7. Polypeptides Precipitated with Anti-AAV2 Serum

in the figure, and incubated at 37°C. One hour later the same cells

E2A expression, which in turn induced the E4-encoded helper effect. To test this idea, we injected E1

definition may become possible once fractionated E4

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, in-

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

Our results show that the helper effect for AAV resides in early region 4, located at the right end of the

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

encoded in the E4 gene cluster. Several distinct

The discovery of a regulatory cascade, E1 → E2A

AAV helper effect, which is just one of many early adenovirus gene functions, will reveal even more ram-

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 Mayor et al. (1977). AAV

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

et al. (1981). DNA was purified from plasmids grown in E. coli strain

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

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

E3 RNAs contained small amounts of E4 RNA, although E4 RNA itself

ering the stringency of the purification procedure.

Preparation of Ad2 DNA Restriction Fragments

Ad2 DNA fragments to be used for microinjection (see Figure 6) were

from a Sma I/Xba I double digest to avoid contamination with Sma I

raphy, extracted three times with phenol, once with chloroform and finally stored in sterile 10 mM Tris–HCl (pH 7.5) at concentrations

pared in this way were judged to be free of neighboring fragments by

than 2% molar contamination.

Microinjection

Our application of the technique of Graessmann et al. (1980) has been

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

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-

fragmented with Bal nuclease in the presence of RNase 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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