Proc. Natl. Acad. Sci. USA
Vol. 78, No. 3, pp. 1925–1929, March 1981
Microbiology

Locations of adenovirus genes required for the replication of adenovirus-associated virus

(adenovirus-associated virus helper function/transfection/gene mapping)

JOHN E. JANIK, MARILYN M. HUSTON, AND JAMES A. ROSE*

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Communicated by Robert J. Huebner, November 13, 1980

ABSTRACT **We have used DNA transfection to identify several regions of the adenovirus genome needed to induce replication of the defective parvovirus, adenovirus-associated virus (AAV). Previous studies have indicated that only early adenovirus functions are needed to aid the replication of AAV. In this report, we demonstrate that three restriction endonuclease fragments of adenovirus DNA are necessary for production of infectious AAV in 293-31 cells (an adenovirus type 5-transformed human embryonic kidney cell line). These fragments map from 28.5 to 29.4, 59.5 to 75.9, and 89.7 to 100 map units on the adenovirus type 2 genome and correspond to the locations of the VAI RNA gene, early region 2, and early region 4, respectively. The 293-31 cell line, which has been found to express early region 1A and 1B proteins, alone is incapable of supporting AAV replication or even AAV DNA synthesis. Additional experiments with adenovirus type 5 host range mutants (group I, *hr*1 and group II, *hr*7) indicate, however, that early region 1A provides an essential function(s) for AAV replication, whereas early region 1B probably does not.**

The adenovirus-associated viruses (AAVs) are defective parvoviruses that contain either a plus or minus single-stranded DNA genome having a molecular weight of 1.4×10^6 (1). They are able to replicate only when cells are also infected with a helper adenovirus (Ad) or herpesvirus (ref. 1; unpublished results). In the absence of a helper virus, AAV adsorption, penetration, and uncoating appear to proceed normally but viral DNA synthesis is undetectable (1, 2). This step, as well as subsequent steps in the replication of AAV, therefore may require, directly or indirectly, one or more helper virus gene products. The possibility that AAV DNA and RNA synthesis might depend upon separate helper factors has been suggested by kinetic data (3). Moreover, the inability of AAV to replicate efficiently in conjunction with certain Ad temperature-sensitive (*ts*) and deletion mutants in different complementation groups (4) suggests at least that expression of multiple Ad genes is a prerequisite for AAV production.

Present evidence indicates that early Ad genes are sufficient to complement AAV replication completely. A DNA-minus *ts* mutant of Ad5, *ts*149, has been shown to support production of AAV to levels comparable to those obtained in coinfections with wild-type Ad5 (5). This mutant maps in early region 5 (18.5–22.0 map units) and expresses the four remaining designated early regions (6, 7). In addition, Richardson *et al.* (8) have recently demonstrated that AAV virion production can be detected when AAV-infected cells are microinjected with poly(A)-selected early Ad2 RNA produced in cells blocked with arabinocytosine (Ara C). Thus, AAV multiplication probably depends upon interactions of proteins that are specified by two or more of four early regions (i.e., regions 1, 2, 3, and 4). Because, for Ad2 and Ad5, the precise map locations of these regions are known, transfection of selected Ad DNA restriction enzyme fragments into AAV-infected cells could provide a direct means for identifying necessary regions.

In the present study, we utilized both gel-purified and cloned fragments of Ad2 and Ad5 DNA in cell transfections and determined their effects on AAV replication by assays of specific immunofluorescent foci and total virus yield. It was found that three separate genomic fragments were needed to induce AAV synthesis in an Ad5-transformed human embryonic kidney cell line (293-31) which expresses only early region 1A and 1B proteins (9, 10). These fragments map from 28.5 to 29.4, 59.5 to 75.9, and 89.7 to 100 map units and correspond to the locations of the VAI RNA gene, early region 2 (which specifies the 72,000-dalton DNA-binding protein), and early region 4, respectively (11, 12). In addition, experiments with two host range mutants of Ad5 [group I, *hr*1 and group II, *hr*7 (13)] suggest that an essential gene(s) is provided by early region 1 and that it lies within the 1A segment.

MATERIALS AND METHODS

Cells and Viruses. The 293-31 cell line (9) was obtained from F. Graham, and Ad2, Ad5, and AAV2 were produced in KB cells as before (14). The AAV stock was three times purified in CsCl and then diluted 1:10 in a glycerol storage buffer (15) prior to heating at 56°C for 15 min to inactivate residual Ad. AAV titers were determined by a fluorescent focus assay. Host range mutants of Ad5 (*hr*1 and *hr*7) were obtained from J. Williams and were propagated and assayed in 293-31 cells.

Preparation of Ad Restriction Endonuclease Fragments. Ad DNA was isolated from CsCl-purified virions by incubation in 1% sodium dodecyl sulfate with 500 μg of proteinase K (E. M. Biochemicals) per ml, followed by three extractions with phenol/chloroform/isoamyl alcohol 25:24:1 (vol/vol). *Eco*RI, *Bam*HI, *Sal* I, *Hin*dIII, *Sma* I [Bethesda Research Laboratories (Rockville, MD)], and *Bal* I (New England BioLabs) digestions were carried out according to manufacturers' recommendations. The Ad DNA restriction endonuclease cleavage maps for these enzymes are shown in Fig. 1 (16). Restriction enzyme fragments were separated in 0.7% (or 1.2% for *Bal* I) agarose in 30 mM NaH_2PO_4/36 mM Tris/1 mM EDTA, pH 8.0, by using a preparative horizontal electrophoresis apparatus [Bethesda Research Laboratories (Rockville, MD)]. The fragments were visualized by long-wavelength ultraviolet illumination after brief ethidium bromide staining (1 μg/ml) and were extracted from the agarose by using a NaI/glass bead procedure (17). Fragments were checked for purity by analytical gel electrophoresis under conditions such that 2–8 ng of DNA could be detected.

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Abbreviations: AAV, adenovirus-associated virus; Ad, adenovirus; FFU, fluorescent-focus unit; *ts*, temperature sensitive.

* To whom reprint requests should be addressed.

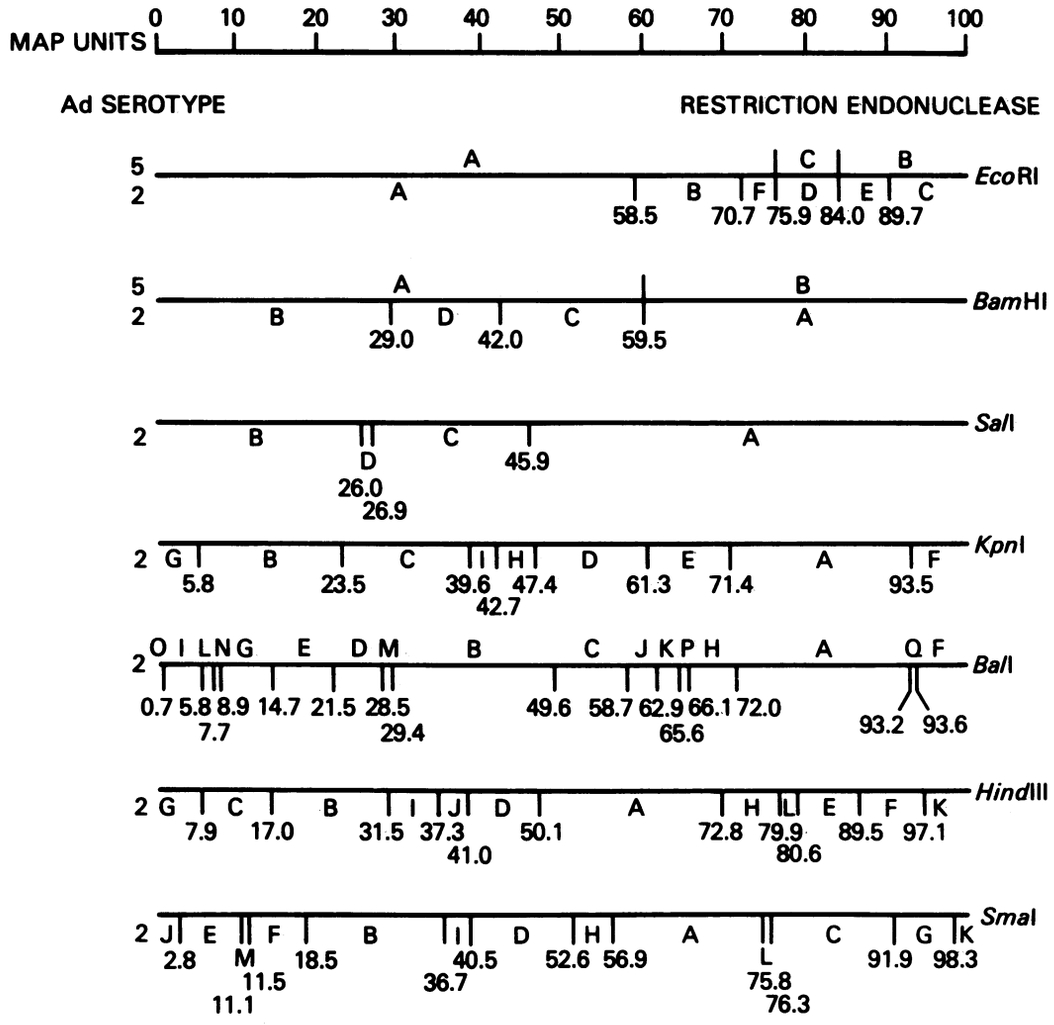

FIG. 1. Ad DNA restriction endonuclease cleavage maps.

Cloning of Ad Restriction Endonuclease Fragments. The *Sal* I C fragment of Ad2 DNA was cloned into plasmid vector pBR322 and propagated in the *Escherichia coli* K-12 strain HB101. Plasmid DNA that contained the *Sal* I C fragment was amplified by chloramphenicol and purified in CsCl/ethidium bromide density gradients. The Ad5 *Bam*HI B′ and Ad2 *Bal* I M fragments were cloned into plasmid vector pBR325 and grown in the same host. Spectinomycin (Upjohn)-amplified plasmid DNA was also purified as above.

Transfections. 293-31 cells were plated in 60-mm petri dishes containing 15-mm circular coverslips, and transfections were performed within 24 hr (75–90% confluence). The cells were infected with AAV at a multiplicity of 25 fluorescent-focus units (FFU) per cell 2 hr prior to transfection. Coverslips were transferred to individual wells of Linbro 24-well plates prior to addition of precipitated DNA. Transfections were performed by using 2.5-μg equivalents (i.e., amounts of each DNA fragment in 2.5 μg of intact Ad DNA) of gel-purified DNA fragments according to the calcium phosphate technique of Graham and Van der Eb (18) followed by a 20% glycerol boost at 4 hr (19). Transfections with the cloned Ad DNA fragments were performed with linearized plasmid DNA because 2-fold higher transfection efficiencies were obtained in comparisons with supercoiled molecules. At 28 hr after transfection, duplicate coverslips were harvested and stained for AAV protein by an indirect immunofluorescence technique. They were then scored at ×250 magnification for typical AAV fluorescence. Antibody specific to the Ad 72,000-dalton DNA-binding protein was obtained from H. Ginsberg.

RESULTS

Pattern of AAV-Specific Fluorescence. Fig. 2 *A* and *C* shows the pattern of typical AAV-specific fluorescence in AAV-infected 293-31 cells transfected with the purified, recombined *Eco*RI fragments of Ad5 DNA. The appearance is identical to that seen in Ad/AAV coinfections. Cells scored as positive in the immunofluorescence assay had bright, predominantly nuclear fluorescence. A striking feature of AAV-infected cells in this and other cell lines is the brilliantly fluorescing discs located in the nucleus which, when examined by phase-contrast microscopy, appear as dark inclusions (Fig. 1*B*). Similar inclusions are not seen in cells only infected with an Ad. Thus, cells producing AAV protein can be identified by phase-contrast microscopy alone.

The 293-31 cell line was selected for this study because of its high efficiency in DNA transfection rescue experiments with Ad *ts* mutants (19). It was produced by transformation of HEK cells with sheared Ad5 DNA (9). DNA sequences from 12% of the left end and 9% of the right end of the viral genome are present in the cells, but only the left end portion of the genome is expressed (10). These sequences specify early region 1A and

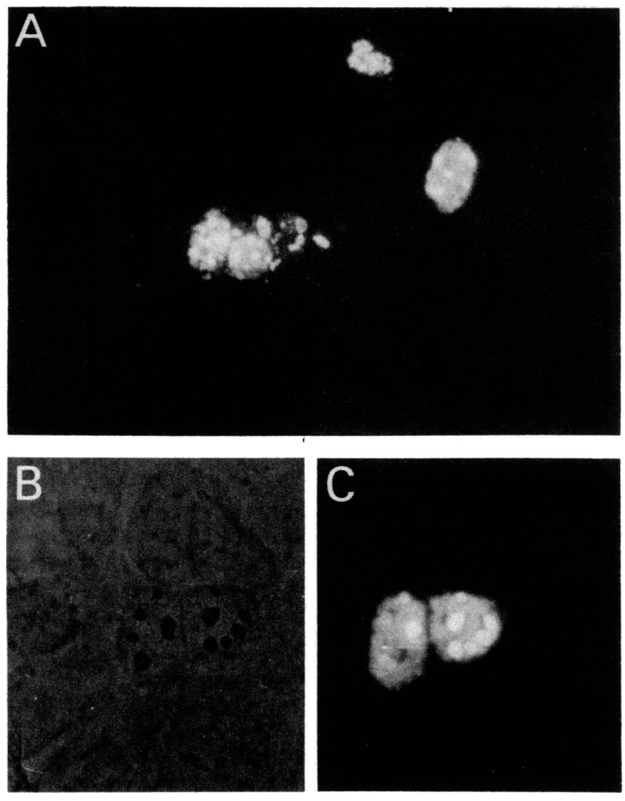

FIG. 2. (*A* and *C*) Indirect immunofluorescence of AAV2 in 293-31 cells after transfection with the three *Eco*RI restriction fragments of Ad5 recombined after purification in agarose. (*B*) Phase-contrast photomicrograph of *C*, showing that fluorescent discs correspond to dark inclusions in the nuclei of the AAV-positive cells. (×250.)

1B products capable of complementing Ad host range and deletion mutants that are defective in 1A and 1B functions (8). The 293-31 cell line by itself will not support AAV DNA or protein synthesis (data not shown).

Transfection Requirements for AAV Replication. With the exception of age of DNA fragments, transfection efficiencies were found to vary from experiment to experiment for reasons not readily apparent. The effect of age of the DNA fragment on transfection efficiency is shown in the bottom half of Table 1. Experiments 2 (247 AAV foci) and 3 (44 AAV foci) were separated from experiment 1 (608 AAV foci) by a period of 1 week and 4 weeks, respectively; the same DNA fragment preparation was used. This suggests a half-life of biological activity of about 1 week. A similar effect was noted with other DNA fragments and occurred without a noticeable change in electrophoretic mobility of the fragments.

The results of transfection experiments in Table 1 demonstrate that at least three regions of the Ad genome are required to complement the replication of AAV. Initial experiments were performed with *Eco*RI-digested Ad5 DNA which gives a simple cleavage pattern comprised of three DNA fragments (Fig. 1). None of the Ad5 *Eco*RI fragments was capable of significant AAV complementation alone, but *Eco*RI A (0–75.0 map units) plus the *Eco*RI B (84–100 map units) distinctly complemented AAV. Complementation was not detected when *Eco*RI A was combined with *Eco*RI C (75.9–84 map units). The amount of the right end of the genome necessary for AAV replication was determined by combining the Ad5 *Eco*RI A fragment with pieces of the right end smaller than the Ad5 *Eco*RI B fragment. To perform these experiments, the Ad2 *Eco*RI C (89.7–100 map units) and the Ad2 *Kpn* I F (93.5–100 map units) fragments were used. Complementation occurred only with the Ad2 *Eco*RI C fragment, indicating that distal sequences of early region 4 (coordinates 91.3/99.3) are required.

Table 1. Detection of three separate regions in the Ad genome that are required for AAV replication

Ad DNA fragment*	No. of AAV-fluorescent cells† Exp. 1	Exp. 2	Exp. 3
5/*Eco*RI A	6	1	0
5/*Eco*RI A + 5/*Eco*RI C	—	0	2
5/*Eco*RI A + 5/*Eco*RI B	99	83	56
5/*Eco*RI A + 2/*Eco*RI C	—	68	45
5/*Eco*RI A + 2/*Kpn* I F	—	—	2
5/*Bam*HI A + 5/*Bam*HI B′ + 5/*Eco*RI B	608	247	44
5/*Bam*HI A + 5/*Bam*HI B′	0	1	1
5/*Bam*HI A + 5/*Eco*RI B	20	28	2
5/*Bam*HI B′ + 5/*Eco*RI B	25	5	0
5/*Eco*RI B	0	1	0

* The restriction endonuclease fragments are identified by Ad serotype followed by restriction endonuclease used and fragment designation indicated in Fig. 1.

† In this and subsequent tables, the number of fluorescent cells represents total foci per coverslip based on the average of duplicate coverslips.

To determine whether the Ad5 *Eco*RI A fragment was providing single or multiple functions, this fragment was cleaved with *Bam*HI, yielding two subfragments extending from 0 to 59.5 map units (*Bam*HI A) and 59.5 to 75.9 map units (*Bam*HI B′). The bottom portion of Table 1 shows the results of experiments demonstrating that the Ad5 *Eco*RI A fragment provides more than one function and that the location of at least one helper function is contained within the *Bam*HI B′ fragment (59.5–75.9 map units) which encompasses early region 2 (coordinates 61.6/75.2).

The results of experiments localizing a function provided by the left half of the Ad genome are shown in Tables 2 and 3. First, the Ad2 *Sal* I A fragment (45.9–100 map units) was used to provide the two regions from the right half of the genome recognized as necessary for AAV replication (Table 2). Although the

Table 2. Further resolution of an AAV helper region within the left half of the Ad genome

Ad DNA fragment*	No. of AAV-fluorescent cells Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
2/*Sal* I A	19	20	18	10	9
2/*Sal* I A + 2/*Sal* I C	195	212	74	75	—
2/*Sal* I A + 2/*Hin*dIII B	—	—	71	46	75

* With the exception of the *Sal* I A fragment, positive foci were not observed when each of the other fragments was transfected alone.

Table 3. Transfection with cloned Ad DNA fragments that contain the VAI RNA and the 72,000-dalton DNA-binding protein coding sequences*

Ad DNA fragment†	No. of AAV-fluorescent cells Exp. 1	Exp. 2	Exp. 3‡	Exp. 4‡	Total AAV yield, FFU‡
5/*Eco*RI B 5/*Bam*HI B′ (cloned) 2/*Sal* I C (cloned)	253	100	51	43	4.8×10^5
5/*Eco*RI B 5/*Bam*HI B′ (cloned)	17	3	0	1	3.3×10^3
5/*Eco*RI B 2/*Sal* I C (cloned)	7	1	2	1	1.6×10^3
2/*Sal* I C (cloned) 5/*Bam*HI B′ (cloned)	1	3	0	0	1.1×10^3
Mock transfection	0	0	0	0	0.9×10^3

* VAI RNA gene, 2/*Sal* I C (coordinates 26.9/45.9) or *Bal* I M (coordinates 28.4/29.5) fragments; 72,000-dalton protein gene, 5/*Bam*HI B′ fragment (coordinates 59.5/75.9).

† Positive foci were not observed in transfections with each fragment alone.

‡ In these experiments, the cloned 2/*Sal* I C fragment was replaced by a cloned 2/*Bal* I M fragment.

Sal I A fragment was capable of AAV rescue to a limited extent when used alone, combinations with either the Ad2 *Sal* I C (26.9–45.9 map units) or the Ad2 *Hin*dIII B (17–31.5 map units) fragment produced 4- to 10-fold increases in AAV yield, whereas neither of the latter fragments demonstrated any helper activity alone. This experiment suggests that a required gene is located in the region between coordinates 26.9 and 31.5, a conclusion further supported by a different approach.

When the *Sal* I C fragment was completely cleaved with *Sma* I or *Bam*HI (cuts at coordinate 29) and transfections then were carried out with the *Sal* I A fragment, the *Sma* I-digested piece retained helper activity but the *Bam*HI-digested piece was inactive, indicating that coordinate 29 may lie within the required gene (data not shown). A similar experiment performed with the Ad2 *Bam*HI A fragment (59.5–100 map units) instead of the *Sal* I A fragment gave comparable results. We therefore concluded that AAV replication may require the VAI RNA gene, which maps between coordinates 28.7 and 29.2, but does not need any of the region encompassed by coordinates 45.9/59.5.

These conclusions were confirmed by the experiments shown in Table 3 in which several cloned DNA fragments were utilized. This series of experiments demonstrates that AAV replication requires one or more early region 4 genes (5/*Eco*RI B fragment) together with cloned DNA fragments that virtually contain only the VAI RNA gene (2/*Bal* I M fragment) and the 72,000-dalton DNA-binding protein gene along with its promotor and leader sequences (5/*Bam*HI B′ fragment) (see experiments 3 and 4 and the yield experiment).

AAV-positive cells sometimes seen in transfections that lack one of the three required Ad DNA regions may be due to small cross contaminations in some gel-purified fragment preparations. The use of cloned DNA fragments might have diminished such foci (Table 3), but a definitive answer will require transfection with a cloned 5/*Eco*RI B fragment.

AAV Requirement for Early 1A Region. Because proteins specified by the early 1A and 1B regions are expressed in 293-31 cells, the importance of these regions in AAV replication was assessed by AAV coinfections with host range mutants of Ad5 that map in either the 1A or 1B regions (mutants *hr* 1 and *hr* 7, respectively). With *hr* 1 in 293-31 cells (permissive cells), the AAV yield was 3 orders of magnitude greater than that from HeLa cells, whereas the *hr*7 mutant gave comparable high yields in both cell lines (Table 4). Defectiveness of *hr*7 in 1B expression in HeLa cells was demonstrated by an absence of Ad5 T antigen-specific fluorescence. These data therefore indicate that region 1A is needed for AAV replication but that 1B is probably not necessary.

Table 4. AAV coinfections with *hr*1 and *hr*7

Virus infection*	Cell line	AAV yield, FFU/10^6 cells
AAV + *hr*1	HeLa	4×10^6
AAV + *hr*1	293-31	5×10^9
AAV + *hr*7	HeLa	1×10^9
AAV + *hr*7	293-31	3×10^9
AAV	HeLa	$1 \times 10^{6\dagger}$
AAV	293-31	$1 \times 10^{5\dagger}$

* Infection multiplicities were: AAV, 50; *hr*1, 15; and *hr*7, 15.

† These values represent residual infectivity from infectious input.

DISCUSSION

The defectiveness of AAV provides a unique system with which to explore biological activities of certain Ad genes. Included are all those genes whose products are directly or indirectly necessary for AAV replication. Because the helper function(s) for AAV replication is expressed early in the Ad cycle [i.e., before or in conjunction with the onset of DNA synthesis (5, 8)], our attention was focused on five regions of the Ad genome known to be transcribed before the onset of viral DNA synthesis. Based on previous findings, however, early regions 3 and 5 could be excluded. Lack of involvement of region 3 was indicated by the fact that nondefective Ad–simian virus 40 hybrids which contain extensive region 3 deletions are as effective as wild-type Ad in helping AAV (R. Dolin, personal communication). Region 5 could be eliminated because *ts*149, a mutant in this region, efficiently helps AAV at the nonpermissive temperature (5). We therefore proceeded to determine directly which remaining early regions were required by assessing the effects of transfected Ad DNA restriction endonuclease fragments on AAV production. The DNA fragments found to be necessary for AAV growth in 293-31 cells map between 28.5 and 29.4, 59.5 and 75.9, and 89.7 and 100 map units on the Ad genome (Fig. 3).

The size and location of two of the required regions correspond to two specific Ad genes. Contained within the segment lying between 28.5 and 29.4 map units (2/*Bal* I M fragment) is the gene for VAI RNA [5.5S RNA (12)]. The adjacent gene that codes for VAII RNA [5.2S RNA (12)] is excluded from this region and presumably cannot substitute for the VAI RNA gene because AAV helper activity was abolished when the 2/*Sal* I C fragment was cleaved with *Bam*HI (cuts the VAI but not the VAII RNA gene). The VAI RNA gene requirement strongly implies that VAI RNA plays a role in AAV replication and provides initial direct evidence that VAI RNA possesses a biosynthetic function. The VA RNAs are not spliced, capped, or polyadenylylated and, although VAI RNA reaches peak levels late in infection, it is detectable less than 3 hr after infection (20). The

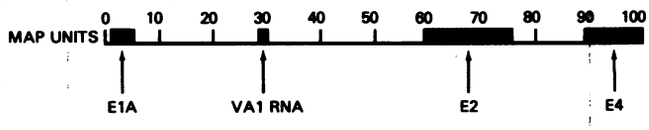

FIG. 3. Schematic representation of Ad2 and Ad5 genetic regions required for AAV multiplication.

DNA (and RNA) sequence for both 5.5S and 5.2S VA RNA predicts that the molecule is able to develop considerable secondary structure through self-complementarity (12). Unlike other adenovirus transcripts, VA RNA is synthesized by cellular RNA polymerase III (20). This enzyme is also responsible for transcription of eukaryotic 5S RNA, tRNA, and small nuclear RNA (21). These various RNAs play roles in DNA synthesis, transcriptional regulation, translation, and, possibly, aligning sites for mRNA splicing (21, 22). Whether VA RNA carries out some analogous function remains to be seen. Recognition of its need in AAV replication, however, now provides a basis for investigating its mode of action.

The second portion of the Ad genome that corresponds to a specific gene is the segment lying between 59.5 and 75.9 map units (5/*Bam*HI B′ fragment). This region encodes the 72,000-dalton DNA-binding protein and includes the observed primary early and late mRNA leader sequences (11). It should be noted that the late 100,000-dalton Ad protein also is specified by sequences in the 5/*Bam*HI B′ fragment (23), but an AAV helper role for this protein can be excluded because (*i*) 5/*Bam*HI B′ fragment lacks all leader sequences for this gene and (*ii*) only early Ad genes appear necessary for AAV synthesis (5, 7). Furthermore, the 72,000-dalton protein is readily detected by specific immunofluorescence in 293-31 cells transfected with the cloned 5/*Bam*HI B fragment (unpublished data). The 72,000-dalton protein is required for Ad DNA synthesis *in vivo* (24) and *in vitro* (25) and apparently is involved in the efficient production of several late Ad proteins (26). Transfection experiments also indicate that AAV DNA synthesis requires the 72,000-dalton protein (unpublished data). This, coupled with recent findings by others which suggest that certain AAV transcripts are greatly diminished in *ts* 125 coinfected cells (4), may reflect a similar multifunctional role for the 72,000-dalton protein in AAV replication.

Concerning the AAV helper function(s) supplied by early region 1A, we suspect that a 1A protein(s) is only needed to induce transcription of other required Ad genes (27). The possibility cannot be excluded, however, that a 1A protein directly supplies some AAV synthetic requirement. In the case of early region 4, which is now thought to code for at least six proteins (28) whose functions are currently unknown, little can be said at present except that one or more of these proteins could be required for AAV replication either directly or indirectly or both.

It should be emphasized that one or more preliminary Ad genetic interactions likely precede the production of any direct-acting AAV helper factor(s). The observed AAV requirement for multiple Ad genes probably reflects this necessity. Therefore, the possibility still remains that AAV replication may only directly require the product of a single Ad gene.

Note Added in Proof. Since this manuscript was submitted, R. A. McPherson in our laboratory noted that a proposed mechanism for Ad mRNA splicing, which involves hybridization to VAI RNA (29), also could apply to AAV mRNA whose nucleotide sequences about the splice junctions (30) have homology to corresponding regions of both VAI and VAII RNA (12).

We thank Jim Williams for helpful suggestions and Alan Gershowitz for assistance with cloning procedures.

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