JOURNAL OF VIROLOGY, Aug. 1980, p. 560-565
0022-538X/80/08-0560/06$02.00/0

Vol. 35, No. 2

Transcripts of the Adenovirus-Associated Virus Genome: Multiple Polyadenylated RNAs Including a Potential Primary Transcript

MICHAEL R. GREEN,[1] STEPHEN E. STRAUS,[2] AND ROBERT G. ROEDER[1]*

Departments of Biological Chemistry and Genetics[1] and Department of Pathology,[2] Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Adenovirus-associated virus type 2 synthesizes four prominent viral transcripts, containing 4.3, 3.6, 2.6, and 2.3 kilobases (kb), in productively infected human KB cells (coinfected with adenovirus type 2). All species are polyadenylated and present in both nuclear and whole-cell RNA preparations, but only the predominant 2.3-kb (and possibly the 2.6-kb) RNA species are found on polysomes. Electrophoretic analyses under denaturing conditions of S1 nuclease-generated and exonuclease VII-generated DNA-RNA hybrids revealed, in each case, four protected DNA fragments which are equal in length (within 50 to 100 nucleotides) to the four S1 nuclease-generated hybrids resolved by electrophoresis under nondenaturing conditions. These results suggest that in the infected cell, abundant adenovirus-associated virus type 2 transcripts are present predominantly (by mass) as unspliced RNAs or, alternatively, they are spliced but contain very short (≤50 nucleotides) leader sequences. That the 2.3-kb RNA represents such a spliced transcript is suggested by exonuclease VII mapping experiments and our more detailed RNA mapping studies (M. R. Green and R. G. Roeder, J. Virol., in press).

The simplicity of parvovirus genomes makes these viruses attractive for studies of eucaryotic gene transcription. Such studies have largely been restricted to adenovirus-associated virus type 2 (AAV2) (for a review, see reference 6). In summary, it was reported that 70% of the AAV2 "minus" DNA strand is transcribed (8, 18) to produce a single stable polyadenylated mRNA of about 20S (0.9×10^6 to 1×10^6 daltons) (7, 9). This RNA was mapped at coordinates 0.18 to 0.88 on the viral genome (7). Recently, it was reported that additional portions of the genome are transcribed and that the corresponding RNA sequences are present in a crude chromatin fraction but not in the mature RNA (13). It has also been shown that the AAV2 genome is transcribed by cellular RNA polymerase II (5).

In apparent contrast to the AAV2 studies, a recent study of the parvovirus H1 transcription program revealed multiple (discrete) polyadenylated H1 RNAs (12). Abundant 2.8- and 3.0-kilobase (kb) and minor 1.45- and 1.30-kb H1 RNAs were found in the cytoplasm and nucleus, whereas a 4.7-kb RNA, corresponding to 95% of the length of the H1 genome, was detected only in the nucleus. Moreover, the 4.7-, 3.0-, and 2.8-kb RNAs were shown to be spliced structures and to contain a common 2.6-kb RNA sequence (body).

To compare the transcription programs of defective and autonomous parvoviruses in a more definitive way, we have characterized AAV2 RNAs with the same high sensitivity and high resolution methods (S1 nuclease mapping) used in our analysis of H1 transcripts. Figure 1 shows a representative example of AAV2 DNA-RNA hybrids fractionated on neutral agarose gels. When adenovirus type 2 (Ad2)-infected KB whole-cell RNA was used, the autoradiogram showed only a single faint band at the 4.8-kb position (upper arrow), representing full-length renatured AAV2 [^{32}P]DNA (lane 2). In contrast, when total cellular RNA from KB cells coinfected with Ad2 and AAV2 (24 h postinfection) was used, four prominent additional bands, corresponding to AAV2 DNA-RNA hybrids, were observed (lane 3). The RNA indicated by the most prominent (lower) band is 2.3 kb. The other (upper) three bands indicate discrete RNAs of about 4.3, 3.6, and 2.6 kb. A minor 3.4-kb DNA-RNA hybrid is also evident just below the 3.6-kb band in lane 3, but its detection was variable (see below). All four abundant AAV2 transcripts were detected in RNA selected on the basis of polyadenylic acid content by oligodeoxythymidylic acid-cellulose chromatography (data not shown).

To gain further insight into the nature and

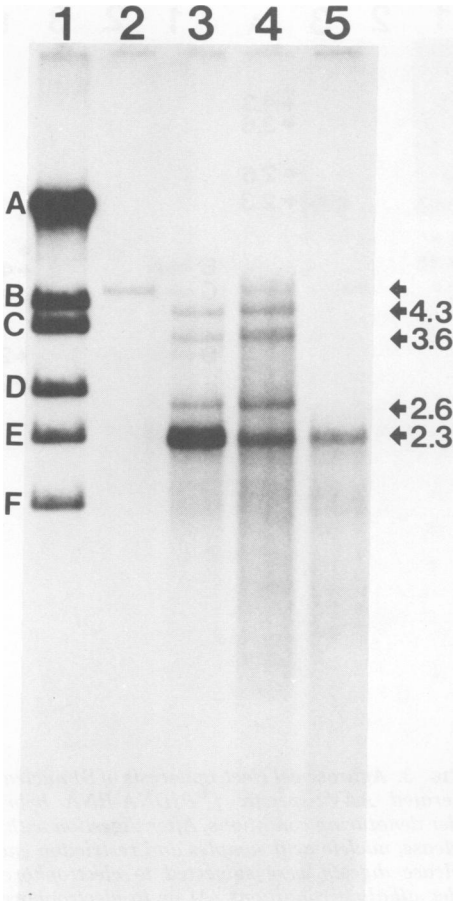

FIG. 1. *Neutral agarose gel electrophoresis of S1 nuclease-generated AAV2-specific [^{32}P]DNA-RNA hybrids formed with RNAs from different subcellular fractions. Our procedures for propagation of AAV2, ^{32}P labeling and extraction of viral DNA, RNA isolation, and S1 nuclease mapping conditions will be described in detail elsewhere (Green and Roeder, in press). We used standard methodology for cellular fractionation (20). Lane 1 contains an EcoRI digest of Ad2 [^{32}P]DNA. Lanes 2 to 5 contain S1 nuclease digestion products of AAV2 [^{32}P]DNA annealed with the following RNAs: lane 2, whole-cell RNA from Ad2-infected KB cells; lane 3, whole-cell RNA from AAV2/Ad2-infected KB cells; lane 4, nuclear RNA from AAV2/Ad2-infected KB cells; lane 5, polysomal RNA from AAV2/Ad2-infected KB cells.*

possible function of the various viral transcripts, nuclear and polysomal RNAs were isolated from 24-h productively infected cells and analyzed by S1 nuclease mapping. It was evident (Fig. 1, lanes 3 to 5) that the major 2.3-kb RNA species was present in all subcellular fractions from infected cells. Because of its relative abundance, polyadenylic acid content, and presence on polysomes, it is highly probable that this transcript functions as the major AAV2 mRNA and that it is identical to the "single" 20S AAV2 RNA reported earlier to be present on polysomes of virus-infected cells (9).

In contrast, the 4.3- and 3.6-kb transcripts were identified in both whole-cell and nuclear RNA fractions (Fig. 1, lanes 3 and 4) but not in polysomal RNA (lane 5), even after very long autoradiographic exposures. Although both the 4.3- and 3.6-kb RNAs are polyadenylated, their absence from polysomes suggests that they do not function as mRNA's.

The 2.6-kb RNA was present in whole-cell and nuclear RNA fractions (Fig. 1, lanes 3 and 4) but was not apparent in polysomal RNA (lane 5) in the experiment shown. However, a longer autoradiographic exposure of Fig. 1 (not shown) revealed a faint band at the 2.6-kb position. This species was detectable in cytoplasmic RNA preparations (not shown), but we cannot rule out the possibility that this resulted from nuclear leakage. Thus, it is plausible that this 2.6-kb RNA functions as a minor mRNA in the polysomes of infected cells, but the present data do not provide definitive information on this point.

Since the S1 nuclease mapping technique is subject to several potential artifacts (2, 3, 12), we confirmed the existence of the four major AAV2 RNAs by an independent procedure. Total cellular RNA samples were denatured by glyoxylation (17), fractionated by size on 1.4% agarose gels, and transferred to diazobenzylmethyl paper (J. C. Alwine, D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl, Methods Enzymol., in press); AAV2-specific RNAs were then detected by hybridization with nick-translated (15) AAV2 [^{32}P]-DNA. As shown in Fig. 2, no discrete bands or nonspecific hybridization background were observed when total RNA from Ad2-infected KB cells was used (lane 2). In contrast, four prominent RNA species were evident when total RNA from KB cells coinfected with AAV2 and Ad2 was analyzed (lane 3). The relative proportions and sizes of these species correspond to those of the AAV2 transcripts detected by S1 nuclease mapping (Fig. 1). The minor 3.4-kb transcript inferred from the S1 nuclease analyses (above) is also visible in Fig. 2 (lane 3).

Because spliced RNAs are a rather ubiquitous feature of eucaryotic cells and their viruses (11, and references therein) and because cells infected with autonomous parvoviruses such as H1 (12) and minute virus of mice (19) accumulate spliced viral RNAs, we next asked whether RNA sequences within each of the four abundant AAV2 transcripts were derived from contiguous or noncontiguous sequences of AAV2

DNA. In these experiments S1 nuclease-generated [^{32}P]DNA-RNA hybrids were analyzed by gel electrophoresis under denaturing conditions. In contrast to our expectations, when AAV2 [^{32}P]DNA-RNA hybrids were analyzed under the denaturing conditions provided either by alkaline gel electrophoresis (16) or by prior glyoxylation (17) of the hybrids, in each case a pattern indistinguishable from that obtained under nondenaturing conditions was observed. Four [^{32}P]DNA segments were produced in each case (Fig. 3A and B, lanes 3). The most abundant fragment was 2.3 kb, and the other prominent species contained 2.6, 3.6, and 4.3 kb. Thus, these data provide no evidence for splicing of abundant AAV2 RNAs. However, the resolution of the gel systems used here is not sufficient to unequivocally establish that the lengths of these DNA fragments are identical to those of the corresponding fragments in the hybrids observed under nondenaturing conditions. Moreover, except for some very minor bands, Fig. 3 does not reveal additional [^{32}P]DNA bands which would be indicative of spliced structures

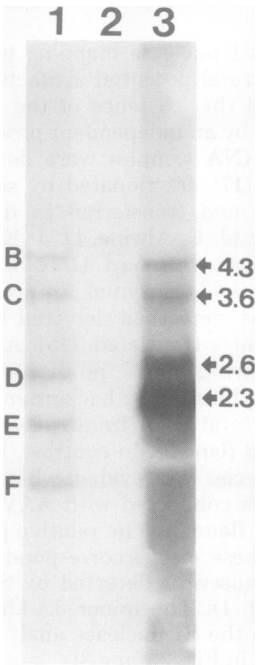

FIG. 2. *Electrophoretic resolution of glyoxylated AAV2-specific RNAs and detection after transfer to diazobenzylmethyl-paper. Samples were glyoxylated, subjected to electrophoresis, transferred, and hybridized to nick-translated AAV2 [^{32}P]DNA as described (Green and Roeder, in press). Lane 1, glyoxylated ^{32}P-labeled Ad2 EcoRI fragments B to D; lane 2, whole-cell RNA from Ad2-infected KB cells; lane 3, whole-cell RNA from AAV2/Ad2-infected KB cells.*

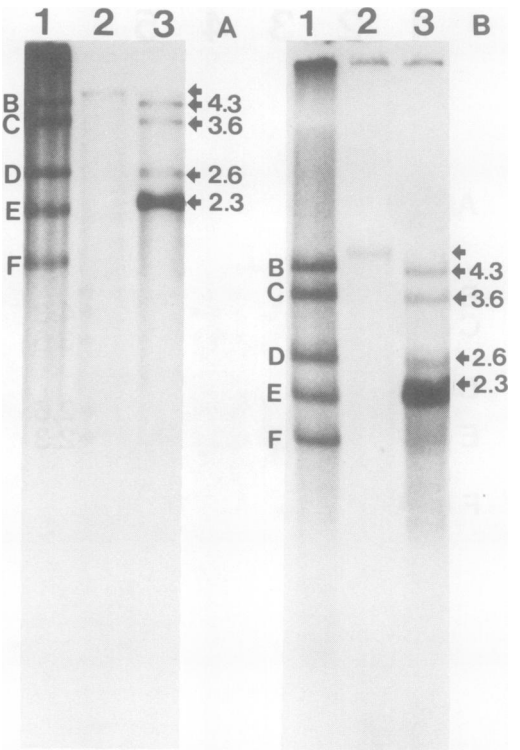

FIG. 3. *Agarose gel electrophoresis of S1 nuclease-generated AAV2-specific [^{32}P]DNA-RNA hybrids under denaturing conditions. After digestion with S1 nuclease, nucleic acid samples and restriction endonuclease digests were subjected to electrophoresis under alkaline conditions (A) or to electrophoresis under neutral conditions after glyoxylation (B). For both (A) and (B), lane 1 contains an EcoRI digest of Ad2 [^{32}P]DNA, and lanes 2 and 3 contain S1 nuclease digestion products from AAV2 [^{32}P]DNA hybridized with whole-cell RNA from Ad2-infected KB cells (lane 2) or from AAV2/Ad2-infected KB cells (lane 3).*

for the AAV2 RNAs. In particular, no low-molecular-weight DNA piece(s) (ca. several hundred nucleotides) is visible in Fig. 3, even after longer exposures. Similarly, when glyoxal-denatured [^{32}P]DNA-RNA hybrids were analyzed on high-percentage acrylamide gels, which readily resolve short DNA fragments, no additional discrete viral DNA-derived oligonucleotides were protected from S1 nuclease digestion by viral RNA (data not shown). These results contrast sharply with those obtained from similar studies of H1 RNAs (12). They indicate that abundant AAV2 RNAs are not spliced at regions between very large stretches of RNA sequences, as found for the large parvovirus H1 transcript (12). They also argue against the possibility (see below) that the AAV2 RNAs contain terminal

"leader" sequences, encoded by noncontiguous DNA regions, which are similar in size (i.e., 100 nucleotides or greater) to those found in the viral RNAs of other animal viruses (11), including autonomous parvoviruses such as H1 (12) and minute virus of mice (19).

Since a very short leader sequence (i.e., 50 nucleotides or less) encoded by a noncontiguous region of the viral genome might not have been detected by the previous experiments, we further searched for splicing of AAV2 RNAs by analyzing exonuclease VII-generated AAV2 [^{32}P]DNA-RNA hybrids. This nuclease digests the protruding 5′ and 3′ ends of DNA in DNA-RNA hybrids, but the single-stranded DNA loops at splice points remain intact (3). Hence, in the case of a hybrid containing a spliced RNA, exonuclease VII digestion should yield a single DNA fragment (upon alkaline gel electrophoresis) whose length is equal to the sum of the lengths of the multiple fragments produced by S1 nuclease digestion plus that of the intervening (spliced out) DNA sequence. In contrast, S1 nuclease- and exonuclease VII-generated [^{32}P]DNA segments derived from hybrids containing unspliced RNAs should be of the same length.

As shown in Fig. 4, exonuclease VII completely digests single-stranded AAV2 [^{32}P]-DNA (lanes 3 and 4) in the presence of EDTA (compare with lane 2), but it does not degrade renatured (double-stranded) AAV2 [^{32}P]DNA (lane 6). The preparation of exonuclease VII used showed no activity when single-stranded circular M13 DNA was the substrate (data not shown), indicating that the observed single-stranded nuclease activity is not due to a contaminating endonuclease. The degradation of single-stranded AAV2 DNA by exonuclease VII is somewhat unexpected since nucleotides 1 to 125 (at both ends of the DNA) can form a "hairpin" (double-stranded) structure (4). Presumably, exonuclease VII can bypass this small hairpin and start digestion at the nearest single-stranded region (at about nucleotide 126; see also reference 10).

A comparison of S1 nuclease- and exonuclease VII-generated [^{32}P]DNA-RNA hybrids is shown in Fig. 5. Exonuclease VII digestion produces four AAV2 [^{32}P]DNA-RNA hybrids (lane 4) which comigrate with the four hybrids generated with S1 nuclease (lane 2). However, the ratio between the 2.3- and 2.6-kb hybrids is significantly altered when the hybrids are formed by exonuclease VII digestion (lane 4). This result suggests the possibility that some or all of the 2.3-kb RNA population is spliced and contains a leader sequence whose 5′ terminus is coincident

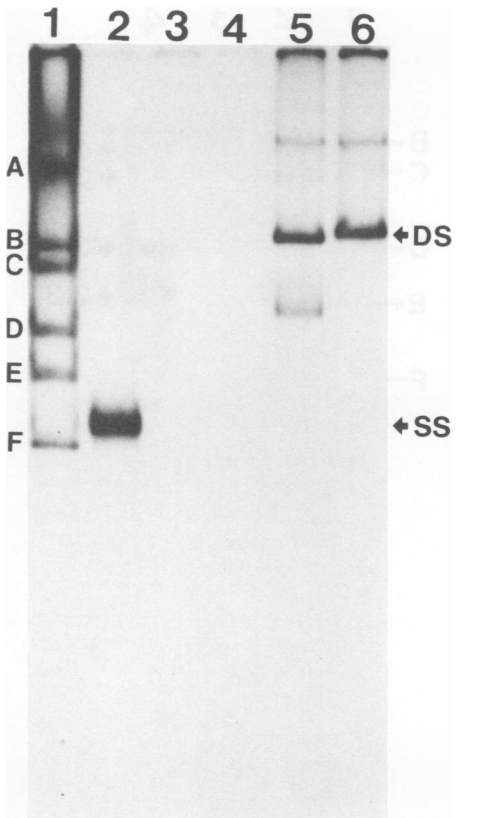

FIG. 4. *Neutral agarose gel electrophoresis of [^{32}P]DNAs after digestion with exonuclease VII. Exonuclease VII digestion conditions were as described (Green and Roeder, in press). Lane 1, EcoRI digest of Ad2 [^{32}P]DNA; lane 2, denatured AAV2 [^{32}P]DNA; lanes 3 and 4, exonuclease VII digests of single-stranded (denatured) AAV2 [^{32}P]DNA digested in the presence of 10 (lane 3) or 25 (lane 4) mM EDTA; lane 5, renatured AAV2 virion [^{32}P]DNA; lane 6, exonuclease VII digestion of double-stranded (renatured) AAV2 [^{32}P]DNA.*

with that of the 2.6-kb RNA. This hypothesis is shown to be correct by more detailed RNA mapping studies with AAV2 DNA restriction fragments (M. R. Green and R. G. Roeder, J. Virol., in press). On the basis of previous demonstrations of the exonuclease VII insensitivity of looped-out DNA regions in DNA-RNA hybrids (3) even when the DNA loop is separated from the RNA terminus by as few as 42 nucleotides (1), these results also argue strongly that the majority of the 4.3-, 3.6-, and 2.6-kb RNA populations are not spliced and do not contain leader sequences encoded at distant (upstream) sites; however, we do not rule out the possibility that a small number of nucleotides may be

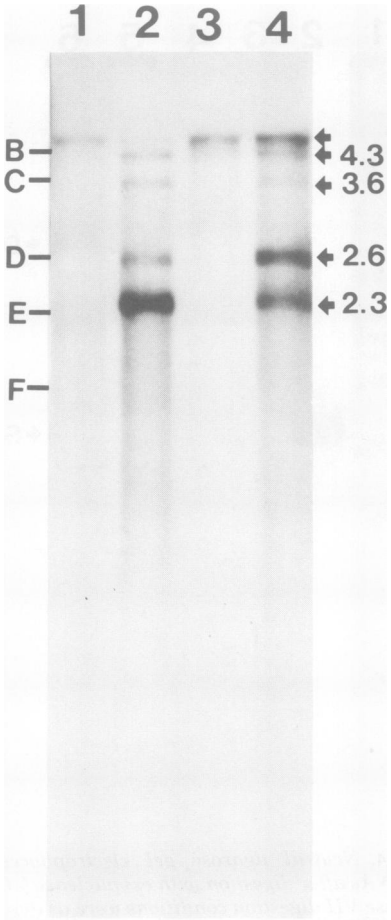

FIG. 5. *Alkaline agarose gel electrophoresis of S1 nuclease- and exonuclease VII-generated AAV2-specific [^{32}P]DNA-RNA hybrids. Details for exonuclease VII mapping are as described (Green and Roeder, in press). AAV2 [^{32}P]DNA was annealed with total RNA from Ad2-infected KB cells (lanes 1 and 3) or from AAV2/Ad2-infected KB cells (lanes 2 and 4); the hybrids resulting from digestion either with S1 nuclease (lanes 1 and 2) or with exonuclease VII (lanes 3 and 4) were subjected to alkaline gel electrophoresis. The positions of the Ad2 EcoRI fragments are indicated by horizontal dashes.*

spliced to any of the RNA termini. A very short leader sequence might not form a stable hybrid under the conditions used; thus, digestion of intervening sequences by exonuclease VII would not be blocked.

The finding of multiple AAV2 transcripts is in excellent agreement with our recent H1 transcription studies, which revealed three predominant H1 RNA species. Significantly, the major 2.8-kb H1 transcript (56% of genome length) appears analogous to the predominant 2.3-kb AAV2 transcript (48% of genome length). Likewise, a less abundant 3.0-kb H1 transcript may be analogous to the 2.6-kb AAV2 transcript.

We have found two AAV2 RNAs, equivalent in length to 75 and 90% of the genome, which are present in nuclei, but not in polysomes. This is similar to our finding that H1 virus encodes a large RNA (94% of the genome) that is found only in the nucleus (12). It is interesting that AAV2-infected cells accumulate two such RNAs, whereas only one large RNA is detectable in H1-infected cells. The present demonstration of these large AAV2 transcripts provides an explanation for the extra RNA sequences associated with the chromatin of AAV2-infected cells (13). Most importantly, the discrete 4.3-kb RNA, which is probably unspliced, is an excellent candidate for a primary transcript of the AAV2 genome. While this remains to be proven, the results presented here suggest that defective parvovirus genomes, like autonomous parvovirus genomes (12), contain a transcription unit comprising over 90% of the viral DNA.

The most striking difference between the H1 and the AAV2 RNA populations is that the most prominent H1 RNAs are present predominantly (by mass) as spliced RNAs containing long (200, 400, and 2,400 nucleotides) RNA segments (leaders) joined to a common 2.6-kb body. Although our results rule out such an extensive (complete) splicing pattern for all the major AAV2 transcripts, we are cautious about concluding that any of the AAV2 RNAs are totally unspliced. Thus, small leader or spliced RNA fragments (less than 50 to 100 nucleotides) located at the ends of viral RNAs would not be detected with the standard S1 nuclease mapping techniques employed, simply because of the inability to detect small size differences (relative to DNA markers) when S1 nuclease hybrids are analyzed under nondenaturing versus denaturing conditions. However, under the hybridization and nuclease digestion conditions employed here, we were unable to detect AAV2 leader sequences via the analysis of [^{32}P]DNA-RNA hybrids on denaturing high-resolution acrylamide gel systems, which proved capable of detecting spliced RNA fragments ($\geq$200 nucleotides) on H1 RNAs (12). The exonuclease VII mapping data presented have provided evidence for splicing, but only in the case of the 2.3-kb RNA, in good agreement with our detailed RNA mapping data (Green and Roeder, in press). Although we do not exclude the possibility of minor populations of spliced counterparts for the two largest AAV2 RNAs, as has been reported in a recent AAV2 transcription study (14), our data suggest that these putative spliced RNAs would be minor

components of the large viral nuclear RNA population. For example, the minor 3.4-kb RNA could result from splicing of the 3.6-kb RNA. However, the 3.4-kb RNA is far less abundant than the 3.6-kb RNA (Fig. 1 and 2). Although our data suggest that AAV2 encodes abundant steady-state RNAs which do not contain transposed leader sequences, a more critical examination of this question, involving more detailed mapping and both DNA and RNA sequence analyses, is necessary. Whether differences in the structure of the steady-state RNAs of autonomous versus defective parvoviruses is in any way relevant to AAV2 defectiveness is an exciting possibility.

This work was supported by American Cancer Society grant NP-284 and Public Health Service grants CA16640 and CA23615 to R.G.R. from the National Cancer Institute and by Public Health Service cancer center support grant CA16217 to Washington University. M.R.G. was supported by an Insurance Medical Scientist Scholarship, and S.E.S. was supported by Damon Runyon-Walter Winchell Cancer Fund Fellowship DRG-112-FT and Public Health Service training grant 5 TO1 AI00459 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Berget, S. M., and P. A. Sharp.** 1979. Structure of late adenovirus 2 heterogeneous nuclear RNA. J. Mol. Biol. **129**:547-565.
2. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721-732.
3. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**: 1274-1278.
4. **Berns, K. I., W. W. Hauswirth, K. H. Fife, and I. S. Spear.** 1978. Terminal structure of adeno-associated-virus DNA, p. 179-192. *In* D. C. Ward and P. Tattersall (ed.), The replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Bloom, M. E., and J. A. Rose.** 1978. Transcription of adenovirus-associated virus RNA in isolated KB cell nuclei. Virology **84**:118-126.
6. **Carter, B. J.** 1978. Parvovirus transcription, p. 33-52. *In* D. C. Ward and P. Tattersall, (ed.), The replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Carter, B. J., K. H. Fife, L. M. de la Maza, and K. I. Berns.** 1976. Genome localization of adeno-associated virus RNA. J. Virol. **19**:1044-1053.
8. **Carter, B. J., G. Khoury, and J. A. Rose.** 1972. Adenovirus-associated virus multiplication. IX. Extent of transcription of the viral genome in vivo. J. Virol. **10**: 1118-1125.
9. **Carter, B. J., and J. A. Rose.** 1974. Transcription *in vivo* of a defective parvovirus: sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and its helper adenovirus. Virology **61**: 182-199.
10. **Chase, J. W., and C. C. Richardson.** 1974. Exonuclease VII of *Escherichia coli*: mechanism of action. J. Biol. Chem. **14**:4553-4561.
11. **Darnell, J. E.** 1978. Implications of RNA:RNA splicing in evolution of eukaryotic cells. Science **202**:1257-1260.
12. **Green, M. R., R. M. Lebovitz, and R. G. Roeder.** 1979. Expression of the autonomous parvovirus H1 genome: evidence for a single transcriptional unit and multiple spliced polyadenylated transcripts. Cell **17**:967-977.
13. **Jay, F. T., L. M. de la Maza, and B. J. Carter.** 1979. Parvovirus RNA transcripts containing sequences not present in mature mRNA: a method for the isolation of putative mRNA precursor sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:625-629.
14. **Laughlin, C. A., H. Westphal, and B. J. Carter.** 1979. Spliced adenovirus-associated virus RNA. Proc. Natl. Acad. Sci. U.S.A. **76**:5567-5571.
15. **Mackey, J. M., K. H. Brackmann, M. R. Green, and M. Green.** 1977. Preparation and characterization of highly radioactive *in vitro* labeled adenovirus DNA and DNA restriction fragments. Biochemistry **16**:4478-4483.
16. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **146**:119-146.
17. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835-4838.
18. **Rose, J. A., and F. Koczot.** 1971. Adenovirus-associated virus multiplication. VI. Base composition of the deoxyribonucleic acid strand species and strand-specific in vivo transcription. J. Virol. **8**:771-777.
19. **Tal, J., D. Ron, P. Tattersall, S. Bratosin, and Y. Aloni.** 1979. About 30% of minute virus of mice is spliced out following polyadenylation. Nature (London) **279**:649-651.
20. **Zieve, G., and S. Penman.** 1976. Small RNA species of the HeLa cell: metabolism and subcellular localization. Cell **8**:19-31.