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Adenovirus-Associated Virus Multiplication

IX. Extent of Transcription of the Viral Genome In Vivo

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Nucleic acid hybridization procedures were used to measure the extent of transcription of adenovirus-associated virus (AAV) deoxyribonucleic acid (DNA) in KB cells in the presence of either adenovirus or herpes simplex virus as the helper. Annealing of AAV ribonucleic acid to AAV DNA was monitored by a hybridization inhibition assay on nitrocellulose filters or by hydroxyapatite chromatography. These experiments confirmed the previous observation that, in the presence of either type of helper virus, only one strand of AAV DNA (the thymidine-rich or "minus" strand) is transcribed in vivo. However, it was found that only 70 to 80% of this strand appears to be transcribed in vivo. Furthermore, studies with minus strands employing hydroxyapatite chromatography and nuclease S_1, which specifically degrades single-stranded DNA, indicated that up to 20% of the minus strand is self-complementary. It seems likely that these self-complementary sequences account for the bulk of that portion of the minus strand (20 to 30%) which is not transcribed in vivo.

It is well established that adenovirus-associated virus (AAV) multiplication is dependent upon adenoviruses (complete helpers) and that herpesviruses (partial helpers) provide some function(s) required for AAV synthesis (2, 3, 6, 12). Both complete and partial helper viruses induce in vivo synthesis of AAV deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (21) and antigen (2, 6). Because the complementary strands of AAV DNA can be separated preparatively (5), it was possible to demonstrate that AAV RNA synthesized in the presence of either a complete or partial helper virus is transcribed from only one DNA strand (the thymidine-rich or "minus" strand) (8, 19). In addition, we have observed that the AAV RNA synthesized in the presence of either helper virus contains similar nucleotide sequences and has a mean molecular weight of approximately 7.5×10^5 (8). An RNA species of this size is equivalent to about 50% of the AAV DNA minus strand of which the molecular weight is approximately 1.5×10^6 (16, 18). Whether one or more AAV RNA species with a molecular weight of 7.5×10^5 might be synthesized in vivo was uncertain because the actual extent of the minus strand transcription was not known.

We now describe experiments designed to measure the proportion of AAV DNA transcribed in vivo. The results of these studies suggest that not more than 70 to 80% of the AAV minus strand, and, as shown before (19), none of the plus strand is transcribed. Furthermore, it was observed during these studies that AAV DNA strands contain self-complementary sequences. This observation has been analyzed in more detail by electron microscopy (Koczot, Carter, Garon, and Rose, *manuscript in preparation*).

MATERIALS AND METHODS

Materials. Hydroxyapatite (Biogel HTP) was obtained from Bio-Rad Laboratories, Richmond, Calif. The source of all other materials has been given (5, 19).

Cells and viruses. KB-3 cells in suspension culture were simultaneously infected with adenovirus-associated virus type 2 (AAV-2) and either adenovirus type 2 [AAV(Ad)-infected cells] or herpes simplex virus type 1 [AAV(HSV)-infected cells] as described before (8, 19). The multiplicity of infection was 10 tissue culture infectious dose units per cell for both AAV-2 and adenovirus type 2 (Ad2) or 1 to 2 plaque-forming units per cell for herpes simplex virus type 1 (HSV-1). When HSV-1 was the helper, the AAV inoculum was heated at 60 C for 10 min prior to infection to inactivate contaminating adenovirus (8).

Isolation of RNA from infected cells. Total RNA was extracted from AAV(Ad)-infected cells at 19 hr or from AAV(HSV)-infected cells at 16 hr after infection by a hot phenol procedure and purified further by treatment with pancreatic deoxyribonuclease (19) and, in some cases, by chromatography on Sephadex

G-25 (4). Purified RNA was stored at −20 C in 0.1 × SSC (0.015 M NaCl, 0.0015 M sodium citrate) at concentrations of 5 to 10 mg/ml.

Preparation of radioactive DNA. The preparation of purified ^{32}P- or ^{3}H-labeled AAV-2 DNA and ^{32}P-labeled, bromodeoxyuridine (BUdR)-containing AAV-2 DNA has been described previously (17, 19, 20). Specific activities of the labeled DNA preparations ranged from 1 to 1.6 × 10^{5} counts per min per μg for ^{32}P- and ^{3}H-DNA and 3 × 10^{4} to 8 × 10^{4} counts per min per μg for ^{32}P-BUdR DNA.

The complementary minus and plus strands of ^{32}P-BUdR AAV DNA were separated by preparative banding in CsCl gradients followed by zonal sedimentation in neutral sucrose gradients as described before (19). Cross-contamination of each complementary strand preparation was less than 5%. The DNA preparations were stored in 0.1 × SSC or 0.01 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), *p*H 8.0.

DNA preparations were fragmented, either by mechanical shear at 50,000 psi by using a Ribi cell fractionator (10), or by sonic treatment for 1 min at 4 C with the microprobe of a Branson sonifier. Both procedures yielded AAV DNA fragments of approximately one-tenth genome size, i.e., 6 to 7*S* (350 to 400 nucleotides long) as determined by sedimentation in alkaline sucrose. Because the fragments produced by sonic treatment were less homogeneous in size than those obtained by mechanical shear, the latter method was usually used to fragment DNA. DNA was denatured either by alkali or heating at 100 C.

RNA-DNA hybridization inhibition on nitrocellulose filters. Method A. Nitrocellulose filters (13 mm) were loaded with 0.03 μg of AAV-2 DNA as described before (8). The filters were incubated in the first step with or without increasing amounts of unlabeled RNA from infected cells in 0.5 ml of 4 × SSC-0.1% sodium dodecyl sulfate (SDS) at 67 C for 24 hr. The filters were then washed in 2 × SSC (50 ml), treated with pancreatic ribonuclease (25 μg in 1 ml of 2 × SSC), and washed again on both sides with 150 ml of 2 × SSC. The filters were then incubated in the second step with 0.3 μg of fragmented, heat-denatured ^{32}P-AAV DNA in 1.0 ml of 2 × SSC-0.1% SDS at 60 C for 20 hr. After this incubation, the filters were washed on both sides with 100 ml of 0.003 M tris(hydroxymethyl)aminomethane, *p*H 9.4 (24).

Method B. The Method B procedure was similar to Method A except that the ribonuclease treatment was omitted, and in the second step only 0.03 μg of DNA was used. In the absence of competing RNA, an input of 0.03 μg of ^{32}P-DNA gave about 35% of the binding observed when the input was 0.3 μg of AAV DNA. The amount of radioactivity bound to blank filters (less than 0.02% of input radioactivity) was subtracted.

DNA reassociation. Reassociation of denatured AAV DNA, either intact or fragmented, was performed at 60 C in 0.14 M phosphate buffer (*p*H 6.8) containing 0.0025 M EDTA. The extent of reassociation was monitored on hydroxyapatite at 60 C as described by Gelb et al. (10). Single-stranded DNA was eluted in 0.14 M phosphate buffer (*p*H 6.8) which contained 0.4% SDS, and duplex DNA was eluted in 0.4 M phosphate buffer. The data obtained were normalized for the proportion of duplex molecules (0 to 6%) remaining after denaturation but prior to any incubation (zero time).

DNA-RNA annealing in solution. Denatured ^{32}P-AAV DNA or ^{32}P-BUdR AAV DNA minus or plus strands were incubated with unlabeled RNA in 0.5 M NaCl at 68 C by the procedure described by Khoury and Martin (14). The concentration of DNA was chosen so that less than 10% of the DNA would reassociate during the incubation. Samples of the incubation mixture (containing 350 to 400 counts per min) were diluted into ice-cold 0.14 M phosphate buffer and stored at 4 C until analyzed on hydroxyapatite. The proportion of nucleic acid hybrid was determined on hydroxyapatite as described before (14), i.e., that proportion of the DNA which bound to hydroxyapatite in 0.14 M phosphate at 60 C but eluted in 0.4 M phosphate.

RESULTS

Inhibition of AAV DNA-DNA hybridization by AAV RNA on nitrocellulose filters. The extent of transcription of AAV DNA was first estimated by measuring, on nitrocellulose filters, that proportion of the AAV DNA-DNA hybridization reaction which could be inhibited by AAV RNA. Up to 35 to 37% of the AAV DNA-DNA hybridization reaction was inhibited by prior incubation of the filters with RNA from AAV(Ad)-infected cells (Fig. 1). Similar amounts of RNA from uninfected cells produced no inhibition of the DNA-DNA reaction. Since only one strand of AAV DNA is transcribed in vivo (20), the observed inhibition of 35 to 37% suggests that not more than 70 to 74% of this strand is transcribed.

Reassociation kinetics of AAV DNA. An alternative procedure to measure the extent of transcription of AAV DNA utilized hydroxyapatite chromatography. However, for these experiments it was necessary to first determine the rate of reassociation of AAV DNA. This was measured by using AAV DNA which was either intact or fragmented to about one-tenth genome size. The rate of reassociation as a function of C_0t (the product of nucleotide concentration and time of incubation expressed as moles of nucleotide × seconds per liter) is shown in Fig. 2. The rate of reassociation of both intact and fragmented AAV DNA was proportional to the DNA concentration used and followed the theoretical curve of a second-order reaction (7). In 0.14 M phosphate, intact AAV DNA had a $C_0t_{1/2}$ (i.e., the C_0t value at 50% reassociation) of 7.4 × 10^{-4}, whereas fragmented strands reassociated with a $C_0t_{1/2}$ of 1.2 × 10^{-3}. The ratio of the $C_0t_{1/2}$ values (i.e., the ratio of the rates of

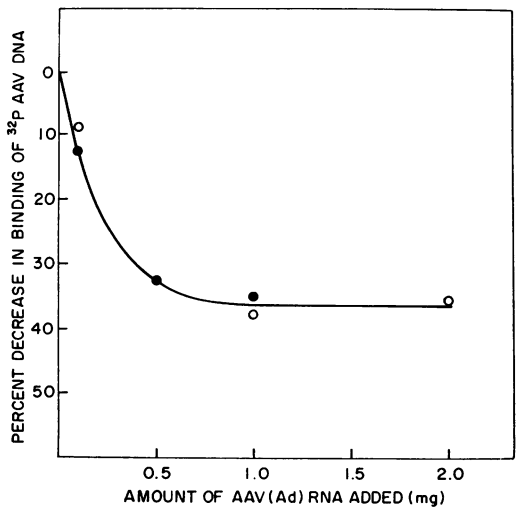

FIG. 1. *Inhibition of AAV DNA-DNA hybridization on nitrocellulose filters by AAV RNA. Filters containing 0.03 μg of AAV-2 DNA were incubated in a first step with unlabeled AAV(Ad) RNA and then in a second step with fragmented, denatured ^{32}P-AAV DNA. Data from two experiments performed by different procedures (methods A and B) are plotted. ○, Method A: input in the second step was 0.3 μg of ^{32}P-AAV DNA. ●, Method B: ribonuclease treatment was omitted and input in the second step was 0.03 μg of ^{32}P-AAV DNA. The results are expressed as the percentage of decrease in binding of ^{32}P-AAV DNA relative to the amount bound in the absence of added RNA in the first step. Counts bound in the absence of RNA were: 592 counts per min, method A, and 209 counts per min, method B.*

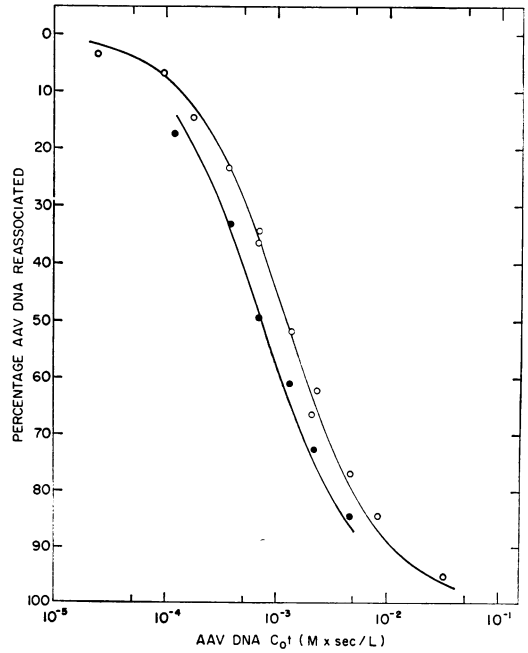

FIG. 2. *Reassociation kinetics of AAV DNA in 0.14 M phosphate buffer at 60 C. Incubation mixtures contained 1.14 or 3.7 × 10^{-4} optical density units of intact, alkali-denatured ^{32}P-AAV DNA per ml (1.2 × 10^5 counts per min per μg) or 8.35 × 10^{-5}, 1.67 or 6.0 × 10^{-4} optical density units of fragmented, heat-denatured ^{3}H-AAV DNA per ml (1.6 × 10^5 counts per min per μg). Samples containing 400 counts per min were removed at intervals, and the percentage of AAV DNA reassociated was determined on hydroxyapatite as described in Materials and Methods. The results are plotted as a function of AAV DNA Cot (moles of nucleotide × seconds per liter). ●, Intact ^{32}P-AAV DNA. ○, Fragmented ^{3}H-AAV DNA.*

reassociation) for the intact strands compared to the fragmented strands was 1.7, which is somewhat lower than the ratio of approximately 3.0 predicted from the data of Wetmur and Davidson (24). However, almost identical values have been obtained for simian virus 40 (SV40) DNA which has a molecular weight similar to that of AAV duplex DNA (14). This may mean either that the molecular weight determination of fragmented DNA in alkaline sucrose is not accurate at low values (6 to 7*S*) or that fragmentation of the intact strands occurred during the reassociation reaction.

Annealing of AAV DNA with AAV RNA in solution. To estimate the extent of minus strand transcription, an amount of fragmented ^{32}P-AAV DNA, of which not more than 10% reassociated during the course of the reaction (Fig. 2), was incubated with increasing amounts of unlabeled AAV RNA synthesized in vivo. The proportion of DNA which formed hybrid molecules with RNA was then determined by their selective retention on hydroxyapatite (Fig. 3). In the absence of any RNA, only 9% of the fragmented DNA reassociated (i.e., bound to hydroxyapatite in 0.14 M phosphate buffer). However, after incubation with RNA from AAV(Ad)-infected cells, a maximum of 40% of the DNA bound to hydroxyapatite, whereas, with RNA from AAV(HSV)-infected cells, at least 35% of the DNA formed hybrids. Because a definite plateau was not clearly reached with the AAV(HSV) RNA, the slightly lower value obtained with this RNA probably reflects a lower level of AAV RNA in these cells rather than a difference in the extent of transcription. When incubated with similar amounts of RNA from uninfected cells, Ad2-infected cells or HSV-1-infected cells, not more than 9% (the value obtained in the absence of any RNA) of the fragmented AAV DNA was retained on hydroxyapatite. It is probable that, in hydridiza-

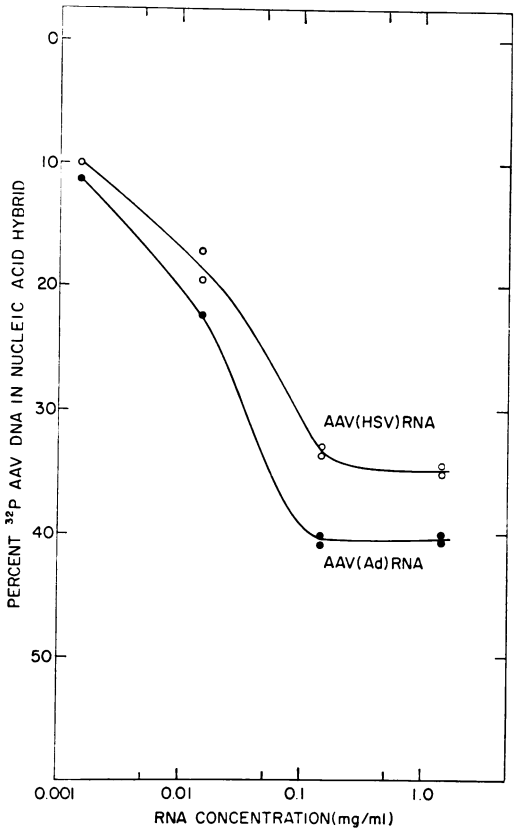

FIG. 3. *Annealing of fragmented AAV DNA in the presence of AAV RNA. Fragmented, denatured ^{32}P-AAV DNA at a concentration of 3 × 10^{-3} μg/ml in 0.5 M NaCl was incubated with increasing amounts of unlabeled RNA from AAV(Ad)-infected cells or AAV(HSV)-infected cells for 2 hr at 68 C. The proportion of ^{32}P-DNA in nucleic acid hybrid was determined on hydroxyapatite. The duplicate points represent reactions using equivalent amounts of different DNA preparations. The results are plotted as the percentage of ^{32}P-DNA contained in nucleic acid hybrid. Each reaction analyzed contained 350 counts per min of ^{32}P-AAV DNA. ●, Unlabeled AAV(Ad)-infected cell RNA. ○, Unlabeled AAV(HSV)-infected cell RNA.*

tion reactions with great AAV RNA excess, the bulk of DNA-DNA reassociation is competed out by DNA-RNA hybridization, and DNA reassociation accounts for far less than this 9% control value. Since only one strand of AAV DNA is transcribed in vivo, the experiment in Fig. 3 suggests that not more than 70 to 80% of this strand is transcribed whether Ad-2 or HSV-1 is the helper.

Annealing of AAV RNA with fragmented minus or plus strands of AAV DNA. The interpretation of the experiment described in Fig. 3 relies upon the previous demonstration (19) that AAV RNA synthesized in vivo hybridizes with only one of the two complementary strand species of AAV DNA. This was reexamined by using hydroxyapatite to detect DNA-RNA hybrids formed with either a plus or minus strand of DNA. In Fig. 4 are shown the results of an experiment in which fragmented AAV DNA minus strands were reacted with AAV RNA. In the absence of any RNA, 8% of the fragmented minus strands reassociated. In the presence of AAV(Ad) RNA or AAV(HSV) RNA, 70% of the minus strands formed hybrids with RNA (Fig. 4). Similar results (not shown) were obtained in other experiments when a sevenfold higher concentration of AAV(Ad) or AAV-(HSV) RNA was used to ensure that sufficient RNA was present to saturate all the transcribed regions of the minus strand. Incubation of AAV RNA with fragmented plus strands of AAV DNA is shown in Table 1. In a 4-hr incubation period, 8% of the fragmented plus strands reassociated in the absence of RNA and 7% in the presence of AAV(Ad) RNA. In a similar period, the same amount of AAV(Ad) RNA was sufficient to anneal with 71% of the minus

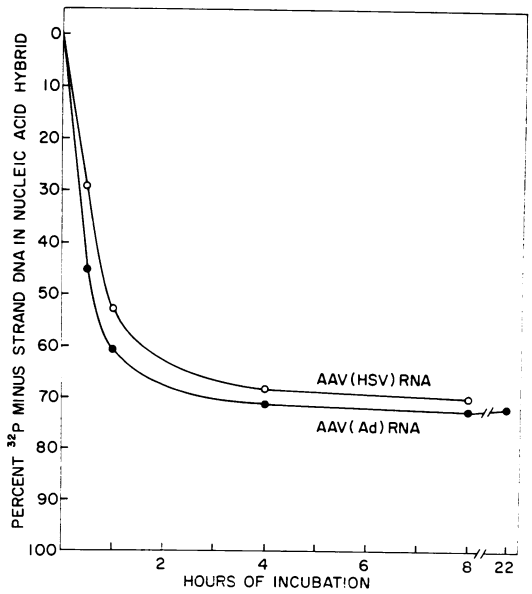

FIG. 4. *Annealing of fragmented minus strands of AAV DNA with AAV RNA. Heat-denatured, fragmented ^{32}P-BUdR AAV minus strand DNA (7 × 10^{-3} μg/ml) was incubated in 0.5 M NaCl at 68 C with unlabeled RNA (0.15 mg/ml) from AAV(Ad)- or AAV-(HSV)-infected cells. Samples were removed at intervals and analyzed as described in Fig. 3. ●, Unlabeled RNA from AAV(Ad)-infected cells. ○, Unlabled RNA from AAV(HSV)-infected cells.*

TABLE 1. *Annealing of separated AAV DNA strands with AAV RNA*

Contents of reaction mixture[a]		^{32}P-DNA in nucleic acid hybrid retained on hydroxyapatite (%)[d]
AAV DNA strand[b]	AAV(Ad) RNA concentration (mg ml)[c]	
Minus strand	0.0	8
Minus strand	0.15	71
Plus strand	0.0	8
Plus strand	0.15	7

[a] Incubations were performed at 68 C in 0.5 M NaCl for 4 hr.

[b] Reaction mixtures contained heat-denatured, fragmented ^{32}P-BUdR-AAV DNA minus strand (7×10^{-3} μg/ml) or plus strand (3×10^{-3} μg/ml).

[c] Unlabeled RNA was extracted from AAV-(Ad)-infected cells 19 hr after infection.

[d] Reactions analyzed contained either 300 counts per min of minus strand DNA or 250 counts per min of plus strand DNA. The percentage of ^{32}P-DNA in nucleic acid hybrid represents the proportion of the DNA which eluted from hydroxyapatite with 0.4 M phosphate.

strand fragments. These experiments confirm that up to 70% of the minus strand, but none of the plus strand, is transcribed in vivo. The separated strands used in these experiments contained BUdR, but the presence of this base analogue does not significantly affect hybridization efficiency as measured on nitrocellulose filters (19). The cause of the small extent (8%) of reassociation of the fragmented, separated strands in the absence of RNA is uncertain. Longer periods of incubation produced no additional DNA reassociation. This reassociation may represent either cross-contamination of the strand preparations or the presence of self-complementary regions within the strands (see below), or both.

Annealing of intact minus strand AAV DNA with AAV RNA. The experiments with fragmented strands of AAV DNA described above show that 70% of the minus strand can hybridize with AAV RNA synthesized in vivo. To be certain that all the minus strands could react with RNA, similar experiments were performed with intact minus strands. However, these experiments revealed an unexpected result. The ^{32}P-BUdR minus strand preparation used was single stranded, at least 90% intact, and less than 5% contaminated with plus strands as determined by the criteria of velocity sedimentation in neutral sucrose and equilibrium banding in CsCl (5). However, in the absence of RNA, 56% of this preparation behaved on hydroxyapatite as molecules with duplex regions (Table 2), and incubation for 2 hr in 0.5 M NaCl did not significantly alter this value. Incubation in the presence of AAV(Ad) RNA showed, as expected, that virtually all of the minus strands now contained duplex regions (Table 2). That 56% of these strands behaved as double-stranded molecules suggested that annealing of self-complementary sequences within the strand had occurred. It is not clear why only 56% of the strands apparently contained duplex regions but may be ascribed in part to damage of the DNA during isolation from virions. Electron microscope studies (Koczot, Carter, Garon, and Rose, *in preparation*), using AAV DNA isolated by different procedures, indicate that at least 70% of the DNA strands contains self-complementary regions. If the minus strands contain self-complementary regions, then breakage of the strands should release both single-stranded and duplex fragments and thus decrease the proportion of DNA which behaves as duplexes on hydroxyapatite. As shown in Table 2, sonic treatment of the minus strands decreased by about one-half the proportion of the DNA which eluted as duplex molecules on hydroxyapatite. Furthermore, incubation of the sonically

TABLE 2. *Properties of AAV DNA minus strands as determined by hydroxyapatite chromatography*

Incubation mixture[a]			^{32}P-DNA in nucleic acid hybrid retained on hydroxyapatite (%)[d]
AAV minus strands[b]	AAV(Ad) RNA concentration (mg/ml)[c]	Incubation time (hr)	
Intact	0.0	0	56
	0.0	2	59
	0.2	2	98
	2.0	2	97
Sonically treated	0.0	0	32
	0.0	2	33
Sonically treated, heat-denatured	0.0	0	8
	0.0	4	12
	0.0	10	13

[a] Incubations were performed in 0.5 M NaCl at 68 C.

[b] Reactions contained 3×10^{-3} μg of ^{32}P-BUdR AAV minus strand DNA per ml.

[c] Unlabeled RNA was extracted from AAV-(Ad)-infected cells at 19 hr after infection.

[d] Each reaction analyzed contained 350 counts per min of ^{3}-P-DNA. The percentage of ^{32}P-DNA in nucleic acid hybrid represents the proportion of the DNA which eluted from hydroxyapatite with 0.4 M phosphate.

treated strands did not result in any significant increase in that proportion which behaved as double strands. This indicates that the single-stranded fragments released by sonic treatment were from noncomplementary regions of the strand. Heat denaturation of these sonically treated strands decreased the proportion of DNA contained in duplex fragments to 8%, and subsequent incubation increased this proportion to not more than 12 to 13%. This residual level of duplex DNA presumably reflects annealing of the self-complementary sequences.

A measurement of the proportion of the DNA strand contained in the self-complementary regions was obtained by digesting the sonically treated, undenatured DNA with the *Aspergillus* enzyme, nuclease S_1, which specifically degrades single-stranded DNA (1, 22). As shown in Table 3, 12 to 14% of the sonically treated AAV minus strand preparation was resistant to degradation by nuclease S_1. In a control experiment, heat-denatured, fragmented SV40 DNA was completely degraded by the same enzyme treatment. This experiment indicates that 12 to 14% of the AAV minus strand was contained in duplex regions. However, hydroxyapatite analysis of the same preparation of minus strands indicated that only 56% of these strands self-annealed (Table 1). Therefore, in these strands, up to 20 to 25% of the strand length may be contained in the self-complementary region. Although a similar analysis of plus strands was not carried out, at least a portion of these strands should also contain self-complementary sequences identical to that of minus strands. The self-complementarity of both plus and minus strands of AAV DNA has been more conclusively demonstrated by electron microscopy (Koczot, Carter, Garon, and Rose, *in preparation*).

TABLE 3. *Degradation of sonically treated, minus strand DNA by nuclease S_1*

DNA[a]	Amount of nuclease S_1 in reaction (μliters)[b]	^{32}P-DNA resistant to degradation (%)
AAV minus strands	0	100
	25	25
	60	14
	70	12
SV40	0	100
	20	1

[a] Nuclease S_1 assays were performed at 50 C for 75 min in reaction mixtures (1.0 ml) containing nuclease S_1 (0 to 70 μliters), 0.03 M sodium acetate buffer (*p*H 4.0), 10^{-5} M $ZnSO_4$, 0.1 M NaCl, 25 μg of fragmented, heat-denatured calf thymus DNA, and either 4×10^{-3} μg (300 counts per min) of sonically treated ^{32}P-BUdR AAV minus strand DNA or 10^{-3} μg (250 counts per min) of fragmented, heat-denatured ^{32}P-SV40 DNA. Reactions were stopped by chilling on ice, and acid-insoluble radioactivity was determined by precipitation with 5% trichloroacetic acid.

[b] The results are expressed as the percentage of ^{32}P-DNA resistant to degradation as calculated from the percentage of acid-insoluble radioactivity remaining after enzyme digestion.

DISCUSSION

The experiments reported here were designed to measure the proportion of AAV DNA which is transcribed in vivo. We have previously shown (8, 19), by analysis in CsCl gradients of AAV DNA-RNA hybrids formed under conditions of DNA excess, that in vivo AAV RNA was synthesized from only one of the two complementary strands of AAV DNA. This observation has now been confirmed (Table 1) by hydroxyapatite chromatography of hybrids formed under conditions of RNA excess. The most direct estimate of the proportion of the AAV minus strand transcribed in vivo was 70% as measured by hydroxyapatite analysis of hybrids formed between fragmented AAV minus strands and RNA from AAV(Ad)- or AAV(HSV)-infected cells (Fig. 3). Experiments performed with fragmented, unseparated AAV DNA strands (Fig. 4) yielded similar results, i.e., up to 80% transcription of the minus strand in AAV(Ad)-infected cells, and at least 70% in AAV(HSV)-infected cells. An alternative procedure (Fig. 1), which measured inhibition of AAV DNA-DNA hybridization by AAV RNA on nitrocellulose filters, also gave an estimate of 70 to 75% for the proportion of the minus strand transcribed in vivo. In summary, these experiments suggested that between 70 to 80% of the AAV minus strand, and none of the plus strand, is transcribed in vivo at 16 to 20 hr after infection whether adenovirus or herpesvirus was the helper. We cannot rigorously exclude the possibility that the remaining 20 to 30% of the minus strand is transcribed only transiently, or at very low levels, thus escaping detection.

The selective retention of intact, separated minus strands of AAV DNA on hydroxyapatite (Table 2) suggested that these strands (and presumably at least some proportion of the plus strands) contain a self-annealed region. Self-annealing of plus or minus strands to form single-stranded circular molecules has now been demonstrated by electron microscopy, substantiating the presence of these self-comple-

mentary regions and showing also that they are at the ends of the AAV DNA strands (Koczot, Carter, Garon, and Rose, *manuscript in preparation*). This implies that RNA transcribed from these terminal sequences should hybridize with both plus and minus strands. However, we have thus far been unable to detect in vivo synthesized RNA which hybridizes with the plus strand of AAV DNA (references 8, 19; Table 1). On this basis, it seems that the self-complementary sequences of AAV DNA are not transcribed in vivo. Furthermore, degradation of AAV DNA minus strands with nuclease S_1 (Table 3) suggests that the self-complementary regions comprise about 20% of the total strand length. If these sequences are not transcribed, they would account for most of that portion of the AAV DNA minus strand which is not transcribed in vivo.

Since the molecular weight of single-stranded AAV DNA is approximately 1.5×10^6 (16, 18), transcription of 70 to 80% of the minus strand would yield RNA species with a total molecular weight of about 1.1×10^6. It was previously shown that AAV RNA synthesized in vivo was mainly composed of molecules having a mean molecular weight of 7.5×10^5 (8). More recently, we have obtained evidence for at least one additional species of AAV RNA with a molecular weight of about 2×10^5 (Carter and Rose, *manuscript in preparation*). If these two species contain different nucleotide sequences, their total molecular weight would approximate that of the transcribed portion of the minus strand.

The extent of in vivo transcription of DNA from several other viruses which have a potential coding capacity similar to that of AAV has been studied by others. For SV40 virus, transcription is equivalent to approximately 100% of a single DNA strand as measured by the same hydroxyapatite procedure used in the present study (14). Other experimental procedures suggest that also for polyoma virus (15) and the single-stranded DNA bacteriophages ϕX-174 (11, 21) and M13 (13), the equivalent of a full strand transcript is synthesized in vivo. Because nothing is known concerning the extent of transcription of other (nondefective) parvoviruses (e.g., minute virus of mice, H-1, or Kilham rat virus), it is not clear whether the observed "incomplete" transcription of AAV DNA is unique to AAV or is a general feature of all parvoviruses. However, it is of great interest that the nontranscribed sequences of AAV DNA appear to represent self-complementary terminal sequences (Koczot, Carter, Garon, and Rose, *manuscript in preparation*) analogous to those found in adenovirus DNA (9). Thus far, only DNA from AAV and their helper adenoviruses has been found to possess this unusual structural feature. This suggests that the nontranscribed sequences of AAV DNA may have some function in common with the terminal sequences of adenovirus DNA. The specific function of these sequences remains to be determined.

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LITERATURE CITED

1. Ando, T. 1966. A nuclease specific for heat-denatured DNA isolated from a product of *Aspergillus oryzae*. Biochim. Biophys. Acta **114**:158–168.
2. Atchison, R. W. 1970. The role of herpesviruses in adenovirus-associated virus replication in vitro. Virology **42**:155–162.
3. Atchison, R. W., B. C. Casto, and W. M. Hammon. 1965. Adenovirus-associated defective virus particles. Science **149**:754–756.
4. Bautz, E. K. F., and E. D. Hall. 1962. The isolation of T4-specific RNA on a DNA-cellulose column. Proc. Nat. Acad. Sci. U.S.A. **48**:400–408.
5. Berns, K. I., and J. A. Rose. 1970. Evidence for a single-stranded adenovirus-associated virus genome: isolation and separation of complementary single strands. J. Virol. **5**:693–699.
6. Blacklow, N. R., M. D. Hoggan, and M. McClanahan. 1970. Adenovirus-associated viruses: enhancement by human herpesviruses. Proc. Soc. Exp. Biol. Med. **134**:952–954.
7. Britten, R. J., and D. E. Kohne. 1968. Repeated sequences in DNA. Science **161**:529–540.
8. Carter, B. J., and J. A. Rose. 1972. Adenovirus-associated virus multiplication. VIII. Analysis of in vivo transcription induced by complete or partial helper viruses. J. Virol. **10**: 9–16.
9. Garon, C. F., K. Berry, and J. A. Rose. 1972. A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Nat. Acad. Sci. U.S.A. **69**:2391–2395.
10. Gelb, L. D., D. E. Kohne, and M. A. Martin. 1971. Quantitation of simian virus 40 sequences in African green monkey, mouse and virus-transformed cell genomes. J. Mol. Biol. **57**:129–145.
11. Hayashi, J., and M. Hayashi. 1970. Fractionation of ØX174 specific messenger RNA. Cold Spring Harbor Symp. Quant. Biol. **35**:171–177.
12. Hoggan, M. D., N. R. Blacklow, and W. P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proc. Nat. Acad. Sci. U.S.A. **55**:1467–1474.
13. Jacob, E., and P. H. Hofschneider. 1969. Replication of the single-stranded DNA bacteriophage M13: messenger RNA synthesis directed by M13 replicative form DNA. J. Mol. Biol. **46**:359–363.
14. Khoury, G., and M. A. Martin. 1972. A comparison of SV40 DNA transcription in vivo and in vitro. Nature N. Biol. **238**:4–6.
15. Martin, M. A., and D. Axelrod. 1968. Polyoma virus gene activity during lytic infection and in transformed animal cells. Science **164**:68–70.
16. Parks, W. P., M. Green, M. Piña, and J. L. Melnick. 1967. Physicochemical characterization of adeno-associated satellite virus type 4 and its nucleic acid. J. Virol. **1**:980–987.
17. Rose, J. A., K. I. Berns, M. D. Hoggan, and F. J. Koczot. 1969. Evidence for a single-stranded adenovirus-associated

virus genome: formation of a DNA density hybrid on release of viral DNA. Proc. Nat. Acad. Sci. U.S.A. **64**:863–869.

18. Rose, J. A., M. D. Hoggan, and A. J. Shatkin. 1966. Nucleic acid from an adeno-associated virus: chemical and physical studies. Proc. Nat. Acad. Sci. U.S.A. **56**:86–92.
19. 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.
20. Rose, J. A., and F. Koczot. 1972. Adenovirus-associated virus multiplication. VII. Helper requirement for viral DNA and RNA synthesis. J. Virol. **10**:1–8.
21. Sedat, J. W., and R. L. Sinsheimer. 1970. The in vivo ØX mRNA. Cold Spring Harbor Symp. Quant. Biol. **35**:163–170.
22. Sutton, W. D. 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochim. Biophys. Acta **240**:522–531.
23. Waarner, S. O., and J. A. Cohen. 1966. A quantitative assay for DNA-DNA hybrids using membrane filters. Biochem. Biophys. Res. Commun. **24**:554–558.
24. Wetmur, J. G., and N. Davidson. 1968. Kinetics of renaturation of DNA. J. Mol. Biol. **31**:349–370.