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Parvovirus RNA transcripts containing sequences not present in mature mRNA: A method for isolation of putative mRNA precursor sequences

(adeno-associated virus)

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ABSTRACT **We report here a method of RNA preparation that may enrich for precursor RNA sequences and the results of an investigation of adeno-associated virus (AAV) RNA transcription that used this method. Whole cells were lysed with detergent and high salt and separated into supernatant and pellet (crude chromatin) fractions. These fractions were then separately deproteinized by proteolytic digestion and phenol extractions. DNA was removed from the preparation by two cycles of pancreatic DNase digestion and phenol extraction. Hybridization analyses of the RNA obtained from AAV/adenovirus-infected KB (human) cells revealed some AAV-specific RNA sequences that were not present in the mature 20S mRNA. These additional sequences were contained in AAV RNA molecules present in the pellet fraction, whereas the 20S AAV mRNA accumulated in the supernatant. A species of AAV-specific RNA (about 22S), which was associated only with the pellet fraction and was labeled only after a short pulse, appeared to have a kinetic relationship with the more stable cytoplasmic 20S mRNA. These putative AAV mRNA "precursors" and precursor sequences were not observed previously when conventional methods were used to obtain RNA from either whole cells or isolated nuclei.**

Parvoviruses, the smallest animal DNA viruses (4600 nucleotide bases in length), appear to code for a single gene and therefore present an attractive system for the analysis of eukaryotic transcription (ref. 1 and references therein). Adeno-associated virus (AAV) transcription and AAV mRNA structure have recently been reviewed (2). Briefly, a single stable 20S polysomal mRNA containing sequences complementary to about 70–80% of the AAV DNA genome is transcribed from the AAV DNA minus strand. The 20S AAV RNA is polyadenylylated at the 3′ terminus and methylated in both the 5′ cap position and internally (B. Moss, A. Gershowitz, and B. Carter, unpublished). Previous experiments provided no firm evidence for any primary transcript that included additional AAV-specific sequences that were not present in the 20S mRNA (3).

We have reinvestigated AAV RNA transcription by using a method of RNA preparation that might be expected to enrich for nascent RNA transcripts. Cells were fractionated by using a modified high-salt/detergent precipitation procedure (4, 5) into a soluble fraction and a crude chromatin fraction. RNA was then isolated by using extensive protease and DNase digestions and phenol extractions. Analysis of the AAV RNA isolated by this procedure revealed that AAV RNA is transcribed from a region between 0.06 and 0.96 of the minus strand that comprises about 10% more of the genome sequence than that represented by the 20S polysomal mRNA. These additional (precursor) sequences are preferentially precipitated in this extraction procedure.

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MATERIALS AND METHODS

Cells and Virus. AAV type 2 (AAV2) was grown in KB (human) spinner cells at 37°C with the wild-type adenovirus 2 (Ad2) as helper (6). The multiplicity of infection with AAV was about 10 tissue culture infectious units per cell and that for adenovirus was 10 plaque-forming units per cell.

Extraction of RNA from Infected Cells. RNA was isolated from infected cells by using a procedure based on previous methods for chromatin isolation (4, 5). Cells were harvested and washed twice with ice-cold phosphate-buffered saline then resuspended in 50 mM Tris·HCl (pH 7.9) at a concentration of approximately 2×10^7 cells per ml. The cell suspension was diluted with 4 vol of a solution containing 0.75% sodium dodecyl sulfate ($NaDodSO_4$) and 12.5 mM EDTA, mixed gently, and allowed to stand at room temperature for 5–10 min. NaCl (5 M) was added to a final concentration of 1.1 M with gentle mixing and the lysate was allowed to stand overnight at 0–4°C. The mixture was centrifuged at $16,000 \times g$ for 30 min in a Sorvall RC-5 centrifuge to pellet the precipitated material. The supernatant fraction was then digested with proteinase K (25 μg/ml; ref. 7) at 40°C for 2 hr. In some cases, if large volumes were involved, the supernatant was first concentrated by ethanol precipitation then resuspended in a buffer containing 10 mM Tris·HCl (pH 7.9), 10 mM EDTA, 0.6% $NaDodSO_4$. The pellet fraction was resuspended in the same buffer with a Dounce homogenizer. Proteinase K was added to 100 μg/ml and the mixture was incubated at 40°C for 2 hr or until the suspension cleared. After the proteinase K digestion both pellet and supernatant fractions were extracted three times with phenol/$CHCl_3$ (50:50, vol/vol) and precipitated with 2 vol of ethanol. The precipitated nucleic acid was resuspended in a minimum volume of buffer (100 mM NaCl/10 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4) and digested with pancreatic DNase at a final concentration of 30 μg/ml. The DNase digestion was performed for 15–20 min at 37°C and the RNA was extracted with phenol/chloroform again and ethanol precipitated. The DNase digestion was repeated with the enzyme at 20 μg/ml, and after phenol/$CHCl_3$ extraction and two successive ethanol precipitations the purified RNA was resuspended in distilled water and frozen at −20°C.

Fractionation of Cells into Nucleus and Cytoplasm. Cells were fractionated into nuclear and cytoplasmic fractions by using either the detergent Nonidet-P40 (NP40) or Dounce homogenization. Briefly, infected cells were washed twice in ice-cold phosphate buffered saline then lysed at 0–4°C in an isotonic buffer containing 0.14 M NaCl, 1.5 M $MgCl_2$, 10 mM Tris·HCl (pH 8.0), and 0.6% NP40. Alternatively, infected cells were lysed by Dounce homogenization in a hypotonic buffer

Abbreviations: AAV, adeno-associated virus; Ad2, adenovirus 2; $NaDodSO_4$, sodium dodecyl sulfate; NP40, Nonidet-P40.

containing 10 mM Tris·HCl (pH 7.5) and 1.5 mM $MgCl_2$. Nuclei were pelleted and washed again with additional buffer. The combined supernatants (cytoplasmic fraction) were adjusted to 0.6% $NaDodSO_4$ and 10 mM EDTA. RNA was then prepared by using the proteinase K, DNase, and phenol/$CHCl_3$ steps as described above. Poly(A)-containing RNA was obtained by passing the RNA through poly(U)-Sepharose (Pharmacia FP) twice. The nuclear fraction was resuspended in 50 mM Tris·HCl (pH 8.0) by Dounce homogenization and the pellet-fraction RNA was prepared exactly as described above for whole cells.

Analysis of Viral RNA in Dimethyl Sulfoxide/Sucrose Density Gradients. RNA was sedimented in dimethyl sulfoxide/sucrose density gradients (36 ml) as described (6).

Preparation of Viral DNA and Restriction Fragments. The preparation of purified AAV2 [^{32}P]DNA and the separation of the complementary plus and minus strands of ^{32}P-labeled, bromodeoxyuridine-substituted AAV2 DNA have been described (8). Specific restriction endonuclease fragments of duplex AAV2 DNA were obtained as described (9) and recovered from gel fractions by electrophoresis. Portions of each fragment preparation were reelectrophoresed to check their purity.

DNA·RNA Hybridization. The hybridization of AAV RNA with ^{32}P-labeled AAV DNA probes was analyzed by using S1 nuclease as described (10).

RESULTS

Sedimentation analysis of RNA in the pellet and supernatant fractions

The distribution of AAV-specific RNA in the pellet and supernatant fractions of infected cells was investigated as shown in Fig. 1. The RNA in an infected cell culture was labeled with [^{3}H]uridine beginning at 16 hr after infection. Half of the culture was harvested after a 30-min labeling period and the other half after 2.5 hr. Each portion of the labeled culture was fractionated according to the procedure detailed above, and the ^{3}H-labeled RNA was extracted from both pellet and supernatant fractions. The purified RNA preparations were then analyzed by velocity sedimentation in denaturing sucrose gradients (Fig. 1).

The ^{3}H-labeled AAV-specific RNA that appeared in the supernatant fraction within 30 min (Fig. 1*B*) was mostly heterogenous and sedimented in the range 4–18 S with a maximum at 10–12 S. After 2.5 hr the ^{3}H-labeled AAV RNA that had accumulated in the supernatant (Fig. 1*D*) comprised both this heterogenous AAV RNA component and a more homogeneous 20S AAV RNA species.

As shown in Fig. 1 *A* and *C*, the ^{3}H-labeled AAV-specific RNA present in the pellet fraction after either a 30-min or 2.5-hr labeling period showed a rather broad profile. However, there was a significant shift of the labeled material to a lower sedimentation rate with increased labeling time. The AAV-specific RNA labeled during the 30-min pulse sedimented with a peak at 18–20 S. The sedimentation coefficient of this peak decreased to 16–18 S when the labeling time was increased to 2.5 hr. The faster-sedimenting shoulder of 21–22 S in the 30-min pulse, although variable in prominence from experiment to experiment, was always present after short pulses but was not present in the RNA preparations labeled for 2.5 hr. However, a 20S AAV-specific RNA accumulated in the supernatant fraction after a 2.5-hr labeling period. The 21–22 S material found in the crude chromatin fraction after a short pulse appears to be relatively unstable and may represent precursors of the cytoplasmic 20 S poly(A)-containing AAV mRNA.

We previously reported (3, 6) that cytoplasmic AAV RNA that accumulated after a 3- to 4-hr labeling period was mainly the discrete 20S species. In the fractionation procedure employed here the supernatant fraction would be expected to include all of the cytoplasmic RNA and some portion of the nuclear RNA. To analyze this further, the RNA in an infected cell culture was labeled with [^{3}H]uridine for 3 hr from 16 to 19 hr after infection. The culture was then fractionated into nucleus and cytoplasm by using the NP40 procedure. The nuclear fraction was further fractionated into a pellet and supernatant fraction by using the high-salt/$NaDodSO_4$ procedure as detailed above. RNA was then extracted from the cytoplasmic fraction and from the nuclear supernatant fraction and analyzed in denaturing sucrose gradients (Fig. 2). The cytoplasmic AAV RNA was mainly the 20 S mRNA (Fig. 2*A*), whereas the bulk of the nuclear supernatant AAV RNA was the heteroge-

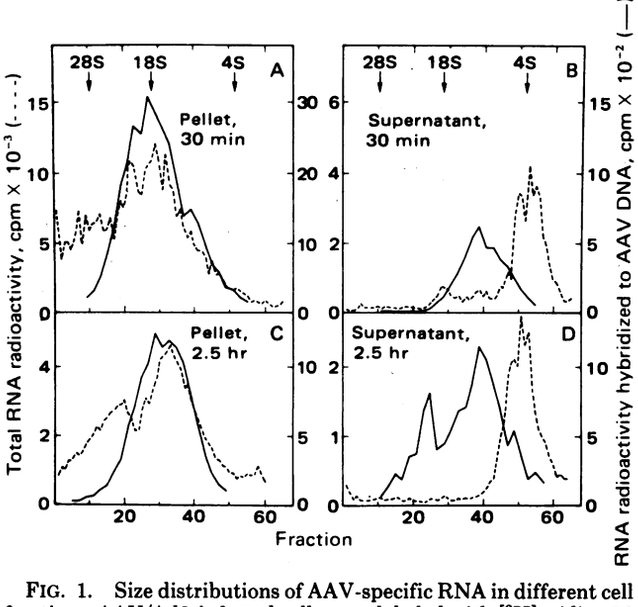

FIG. 1. Size distributions of AAV-specific RNA in different cell fractions. AAV/Ad2-infected cells were labeled with [^{3}H]uridine 16 hr after infection for 30 min (*A* and *B*) or 2.5 hr (*C* and *D*). The cells were fractionated as described in the text into pellet (*A* and *C*) and supernatant (*B* and *D*) fractions. After sedimentation of the samples through dimethyl sulfoxide/sucrose gradients the total [^{3}H]RNA (broken line) and the AAV-specific RNA (solid line) in each fraction were quantitated.

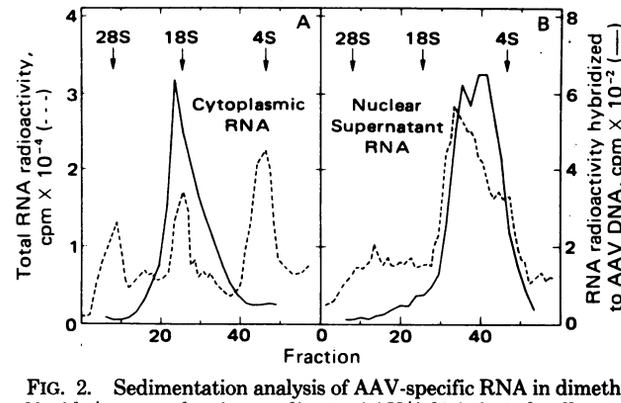

FIG. 2. Sedimentation analysis of AAV-specific RNA in dimethyl sulfoxide/sucrose density gradients. AAV/Ad2-infected cells were labeled with [^{3}H]uridine at 16 hr after infection for 3 hr. The cytoplasmic fraction (*A*) was obtained by treating the cells with NP40 and centrifugation. The nuclear pellet was fractionated by the detergent/high salt procedure to obtain the nuclear supernatant fraction (*B*). Total [^{3}H]RNA (broken line) and AAV-specific RNA (solid line) in each gradient fraction were estimated.

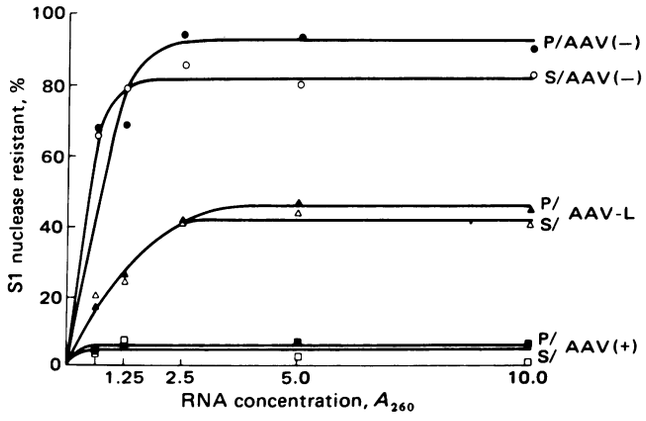

FIG. 3. Extent of S1 nuclease resistance of AAV DNA plus [AAV(+)] and minus [AAV(−)] strands and denatured AAV DNA duplex (AAV-L) after hybridizing with either supernatant (S) or pellet (P) fraction RNA. Conditions of hybridization were as described in the text.

nous 4–18S RNA species (Fig. 2*B*). The origin of this heterogenous RNA that appeared rapidly in the supernatant is unclear (see *Discussion*).

Hybridization of Hirt pellet and supernatant RNA with AAV DNA

KB cells were harvested 16 hours after infection with AAV2 and Ad2 and fractionated by precipitation with detergent and high salt. The RNA was then purified from both the pellet and supernatant fractions by extensive digestion with proteinase K and DNase. To determine the DNA strand specificity, the purified RNA was annealed with the separated plus or minus strands of AAV [^{32}P]DNA. The proportion of [^{32}P]DNA that formed a hybrid resistant to nuclease S1 was determined as a function of RNA concentration. As seen in Fig. 3, the AAV RNA in the supernatant and pellet fractions was complementary to 82% and 92%, respectively, of the minus-strand DNA sequence. When saturating amounts of pellet and supernatant RNA were mixed, approximately 91% of the minus-strand DNA was protected against S1 nuclease (data not shown). These data show that the supernatant AAV RNA sequences are a subset of those in the pellet. Furthermore, the extent of transcription on the minus strand (approximately 90%) as revealed by the reaction with the pellet RNA is significantly greater than that observed (approximately 75%) in previous studies (3, 10). Both the pellet and supernatant RNA showed a small reaction (about 4–5%) with the plus strand (Fig. 3, Table 1). The significance of this result is difficult to assess, but this reaction may reflect contamination of the DNA probe with minus strands.

Fig. 3 also shows the results of annealing the supernatant and pellet RNA fractions with denatured AAV DNA duplexes. In each case the proportion of DNA that was resistant to S1 nuclease digestion (42% and 46%) was approximately half of the value observed when the purified minus strand was used (Fig. 1). This result is consistent with the data obtained by using separated DNA strands as probes, which show only a very small reaction with the plus strand.

Table 1. Mapping of AAV2 RNA

AAV [^{32}P]DNA	DNA resistant to S1 nuclease, %		
	Poly(A)-containing	Supernatant	Pellet
Intact duplex	—	42.0	46.1
Plus strand	—	3.0	4.0
Minus strand	—	79.0	89.0
Bam-B	18.8	30.5	37.1
Bam-A	39.5	43.1	46.8
Hinc-D	19.6	12.8	34.1
Hinc-C	50.9	49.7	51.4

AAV [^{32}P]DNA was hybridized with RNA isolated from infected cells as described in the text. Each value is an average of measurements from at least three separate experiments.

Hybridization of AAV pellet and supernatant RNA with DNA restriction fragments

The genome location of the AAV RNA sequences present in the pellet or supernatant fractions was analyzed by hybridizing the RNA with denatured DNA fragments obtained by cleavage of AAV [^{32}P]DNA duplexes with restriction endonucleases *Bam*HI or *Hin*cII. The fragments used have been mapped previously (11, 12) as shown in Fig. 5. The annealing reactions were performed under conditions in which little or no DNA·DNA hybridization occurred (usually less than 1–4%) so that the proportion of DNA that became resistant to nuclease S1 was a measure of RNA·DNA hybrid formation. If there is little or no RNA hybridization to the plus strand, then for a DNA fragment completely within the transcribed region, the maximum level of protection against nuclease digestion is 50%. Hybridization experiments for each DNA fragment were performed with increasing concentrations of RNA, and the plateau levels of hybridization were determined as for the experiment in Fig. 3. Figs. 3 and 4 show typical hybridization curves with different kinds of ^{32}P-labeled DNA probes from individual experiments and Table 1 summarizes all the results.

Fig. 4 shows hybridization of the RNA fractions with the *Bam*-A and *Bam*-B fragments. Both these fragments showed

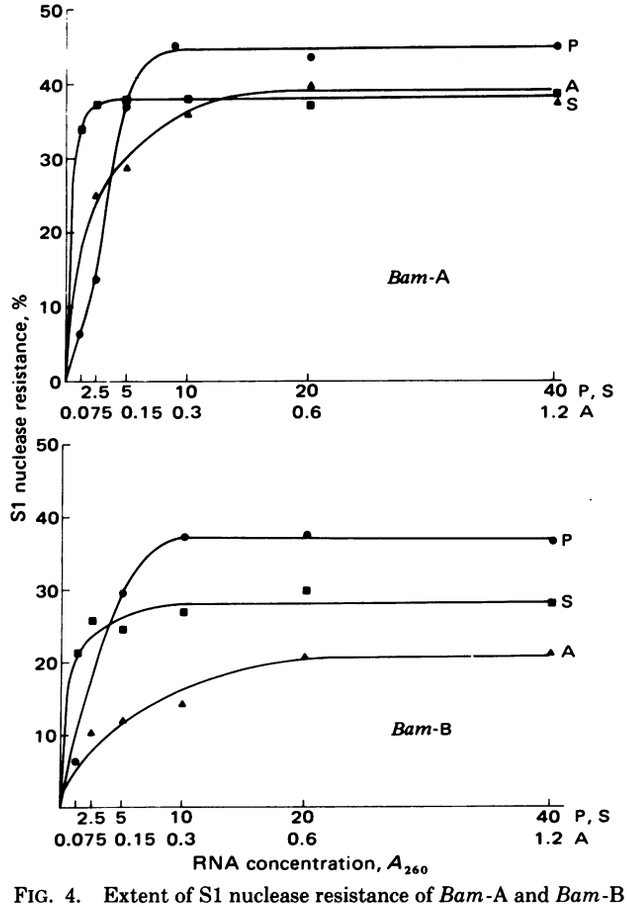

FIG. 4. Extent of S1 nuclease resistance of *Bam*-A and *Bam*-B fragments after hybridization with pellet (P), supernatant (S), or poly(A)-containing (A) RNA.

less than 50% hybridization with either the pellet or the supernatant RNA. Furthermore, for both DNA fragments, the extent of hybridization with the pellet RNA was greater than with the supernatant RNA. Similar experiments were also performed with restriction fragments *Hin*c-C and *Hin*c-D (restriction map shown in Fig. 5), and the data are summarized in Table 1. Approximately 50% of the *Hin*c-C fragment hybridized with both pellet and supernatant fractions, and therefore this region of the genome, 0.31–0.51 map units, is completely within the transcribed regions. The *Hin*c-D fragment, which contained the right-hand end of the genome, hybridized much less than 50% with pellet RNA (34.1%) and even less with supernatant RNA (12.8%). These values were consistent with those obtained with the overlapping fragment *Bam*-A. These results also show that the AAV RNA in the pellet fraction contained sequences complementary to the DNA minus strand in addition to those present in the supernatant RNA. The additional AAV RNA sequences mapped near the left and right ends of the viral genome.

Hybridization of AAV poly(A)-containing RNA with AAV DNA restriction fragments

The supernatant RNA was isolated from cells 16 hr after infection and fractionated on a poly(U)-Sepharose column. The poly(A)-containing RNA was then annealed with the *Bam*-A, *Bam*-B, and *Hin*c-D fragments. We have previously shown (3) that the poly(A)-containing AAV RNA consists only of the 20S AAV mRNA. The results of the hybridization experiment are shown in Table 1. The *Bam*-A and *Hin*c-D fragments each showed approximately the same extent of transcription on the right-hand end of the genome with the poly(A)-containing RNA as with the supernatant RNA. For example, *Hin*c-D, which is 14% of genome length, is protected to 12.8% and 19.6% after hybridization with supernatant and poly(A)-containing RNA fractions, respectively. This represents a difference of less than 1% of the genome length between the two RNA fractions. At the left-hand end of the genome, the hybridization plateau obtained with the poly(A)-containing RNA and the *Bam*-B fragment (19%) was significantly less than that obtained with either the pellet (37%) or supernatant (30%) RNA preparations. As expected, no difference in the extent of protection of *Hin*c-C (approximately 50%) was found between any of the RNA fractions.

Genome location of AAV RNA transcripts

The hybridization data are summarized in Table 1. The simplest transcription map that can be drawn from these data is shown in Fig. 5. The genome orientation and direction of transcription shown were determined previously (13). If it is assumed that the AAV RNA in the pellet represents a single transcript of a contiguous region of the minus strand, then this region spans from approximately 0.06 map units at the left to 0.96 map units to the right on the minus strand. The size of the transcribed regions for pellet or supernatant RNA estimated by summing the fragment data agree well with the values obtained from separated minus strands or denatured duplex molecules. This agreement shows the internal consistency of the data (Table 1). Also, the data obtained by using the poly(A)-containing RNA prepared from the Hirt supernatant RNA fraction are consistent with the values previously obtained with poly(A)-containing RNA obtained by isolation with NaDodSO$_4$/hot phenol (11).

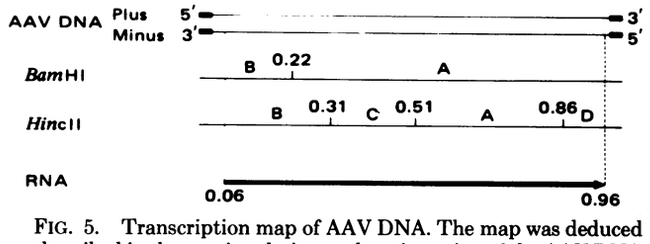

FIG. 5. Transcription map of AAV DNA. The map was deduced as described in the text in relation to the orientation of the AAV DNA and *Bam*H-I and *Hin*cII restriction endonuclease maps as shown.

However, the regions of the genome represented in the supernatant RNA and the poly(A)-containing RNA cannot be readily located accurately on the genome in this study unless it is assumed that each contains a single transcript from a contiguous region. In view of the recent discovery of RNA splicing, this assumption is not necessarily justified.

This study suggests, regardless of the reservations about the exact location of the poly(A)-containing RNA, that the pellet fraction RNA contains a precursor(s) of the supernatant and of the poly(A)-containing AAV mRNA, and that the initial AAV RNA transcript spans at least from 0.06 to 0.96 map units on the AAV genome.

DISCUSSION

Previous studies on AAV RNA (3, 10, 11) did not offer firm evidence to support the presence of any primary transcript that included sequences complementary to the AAV DNA in addition to those present in the mature 20S mRNA [poly(A)-containing polysomal RNA]. This may be due to the short half-life of the precursor RNA or to the use of the hot NaDodSO$_4$/phenol extraction method (14). In order to maximize the sensitivity of detection of initial transcripts we used a procedure for RNA isolation that might be expected to enrich for nascent transcripts. Nascent transcripts would be expected to have a stretch of RNA on the 3′ end base-paired with the DNA template at the time of cell harvest. This hypothesis was the basis of the choice of Marko and Butler's method (4) to precipitate cellular chromatin as a procedure to fractionate nuclei from AAV-infected KB cells. When the isolated nuclear fraction was treated with detergent and high salt (4, 5) it was found that some extra AAV-specific RNA sequences that were not present in the mature 20S mRNA were precipitated along with the cellular chromatin and thus were greatly enriched in the pellet fraction. Because this fraction of AAV-specific RNA was associated with the crude chromatin fraction, such a fractionation procedure could be performed on whole cells without first isolating the nuclei. This should reduce the chance of RNA degradation due to nucleases. In contrast, the mature poly(A)-containing 20S AAV mRNA appears to remain in the supernatant fraction. Thus, the procedure we have described allows a simple and rapid fractionation of mature mRNA molecules from potential precursor transcripts with minimal degradation due to nucleases that may be activated by cellular disruption.

The detergent/high-salt precipitation procedure has previously been used (5) to precipitate cellular chromatin from infected cells in order to separate it from viral DNA. When purified exogenous ^{32}P-labeled AAV virions or AAV DNA duplex were added to the infected cell suspension prior to cell lysis with NaDodSO$_4$, almost all the radioactivity was found in the high-salt supernatant fraction with only a small fraction associated with the pellet.

Extensive digestion of the supernatant and pellet fractions with proteinase K prior to extraction with phenol/CHCl$_3$ reduces degradation by nucleases (7), allows better deproteinization, and reduces loss of chromatin and chromatin-associated material due to trapping by the large amounts of denatured protein in the interphase. The efficiency of the DNase treatment is demonstrated by the observation that the RNA preparation hybridized only with AAV minus strand DNA.

The analysis of the size of AAV RNA in the pellet and supernatant is generally consistent with previous analyses (3, 6). Specifically, in a 30-min labeling period most of the RNA synthesized is heterogeneous in size and is found mainly in the pellet or cell nucleus. However, the fraction of AAV-specific RNA that sedimented at about 22 S is relatively much higher in the pellet fraction than that previously found in the cell nucleus. In longer labeling periods the 22S material is lost and the spectrum of labeled material in the pellet shifts to a lower sedimentation value with a concomitant accumulation of the 20S AAV mRNA in the supernatant or cytoplasm. The nature of the heterogenous 4–18S AAV RNA that accumulated in the supernatant is still not clearly defined. This RNA was also observed in previous studies (3, 6) and was shown to be deficient in poly(A) and to accumulate initially more rapidly but be less metabolically stable than the 20S mRNA. Because this RNA accumulates much faster than 20S RNA, it does not appear to result from cleavage or processing of the putative 20S precursor. Other evidence suggests that part of this RNA may derive from transcription of aberrant deleted AAV DI (defective-interfering) genomes (C. A. Laughlin and B. J. Carter, unpublished).

The hybridization experiments reported here indicate that there may be post-transcriptional cleavage of AAV RNA and that the primary transcript spans at least from 0.06 to 0.96 map units of the AAV genome. This is supported by sedimentation analysis of the RNA, which showed that a large AAV-specific RNA (about 22S) was labeled in the pellet fraction after a short pulse and was greatly reduced after a 2.5-hr pulse. However, a 20S AAV-specific RNA accumulated from a negligible level in a 30-min pulse to a discrete peak at 2.5 hr in the supernatant or cytoplasmic fraction of infected cells. If the RNA in the pellet is indeed a precursor of the AAV 20S mRNA, then post-transcriptional processing (i.e., cleavage or splicing or both) must occur in both the 5′- and 3′-terminal regions of the RNA. The precise location of the regions where such processing would occur requires further analysis. However, it is clear from the data in Table 1 that, at the 5′ end, processing must occur to the left of the *Hin*cII site at 0.31 map units. At the 3′ end there must be processing in the region bounded by the *Hin*c-D fragment—i.e., to the right of 0.86 map units.

The promoter for AAV transcription cannot be precisely located in the current work, but it is presumably at or to the left of 0.06 map units. The first 145 nucleotides of AAV DNA at either end comprise the inverted repetition and the first 125 nucleotides of this sequence are partially palindromic and can fold into a base-paired, T-shaped hairpin (15). Initiation of AAV DNA is believed to occur from the 3′ end of either DNA strand with this 125-nucleotide palindrome as a self-priming structure (15–17). This suggests that the AAV DNA replication origin at the left-hand genome terminus (Fig. 5) may be within 120 to 130 nucleotides of the AAV promoter. A similar argument suggests that AAV transcription may terminate at, or close to, the right hand replication origin.

It is not yet clear why the "precursor" AAV RNA sequences are found in the detergent/high-salt precipitation pellet. Several possible explanations can be suggested.

(*i*) The transcription template may be integrated into the host cell genome but AAV transcription complexes can be solubilized by detergent treatment of infected cell nuclei (C. A. Laughlin and B. J. Carter, unpublished).

(*ii*) The AAV transcription template might be a higher molecular weight species of AAV DNA such as the concatemeric AAV replicating intermediates (16, 18). Most of these, however, do not precipitate in the Hirt procedure either with or without (19) prior proteolytic digestion. This implies that the AAV transcription templates may be distinguishable from the bulk of AAV replicating intermediates.

(*iii*) It is possible that the AAV transcription template is closely associated with the cellular chromatin. Alternatively, the nascent or "precursor" RNA may be chromatin-associated, perhaps because of the location of the RNA processing apparatus.

The association of nascent AAV RNA transcripts with the cellular chromatin is of interest in view of recent suggestions that both DNA replication and viral assembly of the autonomous parvoviruses *Lu*III (20, 21) and H-1 (22, 23) are also closely associated with cellular chromatin.

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