JOURNAL OF VIROLOGY, Nov. 1979, p. 394-403
0022-538X/79/11-0394/10$02.00/0

Vol. 32, No. 2

Synthesis and Processing of Adenoviral RNA in Isolated Nuclei

V. W. YANG AND S. J. FLINT*

Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08544

Received for publication 25 May 1979

Adenoviral RNA sequences synthesized in nuclei isolated during the late phase of productive infection comprise, besides virus-associated, VA, RNA, five major species, ranging in size from approximately 13S to 55S. The latter RNA species is of the length predicted if the major transcriptional unit expressed during the late phase were completely copied in vitro. Some 30% of the RNA sequences labeled in vitro are polyadenylated, and about one-third of the polyadenylated RNA is virus specific. Hybridization analysis of the sequences immediately adjacent to polyadenylic acid in late RNA labeled in isolated nuclei suggests that polyadenylation in vitro occurs at the same sites recognized within the cell. The polyadenylic acid-containing viral RNA sequences made in isolated nuclei are found in three major species of RNA, sedimenting at approximately 28S, 18S, and 13S. These sizes are remarkably similar to those reported for late mRNA species, suggesting that additional processing steps can occur in isolated nuclei. Hybridization of RNA to *Xho*I fragments of adenovirus type 2 DNA transferred to nitrocellulose filters reveals that sequences complementary to the region from 22.0 to 26.5 units present in 55S RNA are absent from all smaller species, suggesting that the smaller RNA species labeled in isolated nuclei are generated by splicing. The splicing events necessary to generate the 5′ leader segment common to the majority of late adenoviral mRNA species are shown to be performed correctly in isolated nuclei.

The expression of adenoviral genetic information during productive infection of human cells is an orderly process, mediated by mechanisms analogous to those operating in normal, uninfected cells (see reference 14 for a review). A great deal is now known about the number, location within the adenoviral genome, and structure of viral mRNA species synthesized during different phases of the productive cycle (3, 4, 8, 9, 20, 25, 26). In addition, a late transcription unit from which the precursor to most of the late adenoviral mRNA species is synthesized has been well characterized and is illustrated in Fig. 1 (1, 40, 44). In many ways, then, adenovirus-infected cells, especially those harvested after initiation of viral DNA synthesis, seem to constitute an ideal source from which to isolate subcellular systems that retain transcriptional activity in vitro. By the late phase, infected cells contain very large amounts of viral DNA, which is transcribed so actively that about half the newly made, non-nucleolar RNA is adenovirus specific (2). Their main advantage, however, comes from the opportunity, provided by our detailed knowledge of adenoviral gene expression, to assess the fidelity of transcription in vitro.

Nuclei isolated from adenovirus-infected cells have been reported to retain the capacity to synthesize, apparently correctly, the small virus-associated, VA, RNA species (18, 36, 37, 42), whose genes are transcribed by RNA polymerase form III. RNA sequences corresponding to those present in late mRNA species and their precursor can also be transcribed by form II RNA polymerase in isolated nuclei (18, 40, 41). Here we describe characterization of adenoviral RNA species that are complementary to the late transcriptional unit made in isolated nuclei and show that they are generated by in vitro polyadenylation and splicing.

MATERIALS AND METHODS

Cells and virus. HeLa cells were maintained at densities of 2×10^5 to 5×10^5 cells per ml in suspension cultures in RPMI 1640 medium (GIBCO) supplemented with 5% fetal bovine serum (Flow Labs). Infections with adenovirus type 2 (Ad2) were performed at a multiplicity of 40 PFU/cell, and virus preparations were titrated on HeLa cells as described by Williams (43).

RNA synthesis in isolated nuclei. Nuclei were prepared from infected cells swollen in hypotonic buffer similar to that described by Vennström and Philipson (37), with dithiothreitol replaced by β-mer-

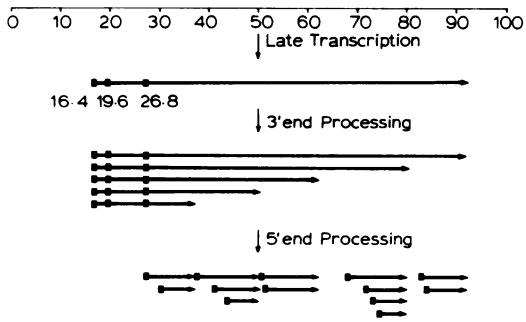

FIG. 1. *Synthesis of type 2 adenoviral mRNA species. The adenoviral genome is represented by the solid horizontal line, 0 to 100 units, at the top of the figure. During the late phase of infection, the r strand is transcribed from 16.4 units (44) to the right hand end of the viral genome (15a) to produce the large transcript shown (1, 40). Poly(A) is added at five sites within this transcript (16, 25, 26), and the three segments that constitute the 5'-terminal segment common to all the major late mRNA species (3, 8, 12, 21) are spliced to each other and to the body of each mRNA, as shown. Polyadenylation is believed to precede splicing (27).*

captoethanol. Nuclei were resuspended at concentrations of 3×10^7 to 6×10^7 nuclei per ml in 0.03 M Tris-hydrochloride (pH 7.9) containing 0.06 M $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM β-mercaptoethanol, and 25% glycerol. Incubation was at 30 to 37°C for 0.5 to 1.0 h in the presence of 1 mM ATP and 0.25 mM each CTP, GTP, and UTP. Also present were either [^{3}H]UTP (35 Ci/mmol; New England Nuclear Corp.) or [α-^{32}P]UTP (290 Ci/mmol, New England Nuclear Corp.). In some reactions, 1 part in 4 or 5 of the CTP was replaced with Hg-CTP (PL Biochemicals).

Isolation and purification of RNA. At the end of the transcription reaction, RNA was isolated as described by Seeburg et al. (32). Nuclei were lysed by homogenization in 0.01 M Tris-hydrochloride (pH 7.4) containing 1 mM EDTA and 7 M guanidine hydrochloride. After addition of 0.5 g of CsCl per ml and heating at 68°C for 5 min, the mixture was layered over a 3-ml cushion of 5.7 M CsCl in cellulose nitrate SW41 tubes and centrifuged at 50,000 × g for 20 h at 4°C. The RNA pellet was dissolved in 0.01 M Tris-hydrochloride (pH 7.4) containing 1 mM EDTA, an equal volume of 0.02 M Tris-hydrochloride (pH 7.4) containing 7 M urea, 0.3 M NaCl, 2 mM EDTA, and 2% sodium dodecyl sulfate was added, and the mixture was extracted twice with phenol-$CHCl_3$ (1:1, vol/vol). The RNA was then precipitated with 2 volumes of ethanol and stored at −20°C. In some experiments mercurated RNA was selected by chromatography on sulfhydryl-agarose columns as described in detail elsewhere (Yang and Flint, submitted for publication).

Sucrose gradient sedimentation. Sedimentation was performed in 15 to 30% (wt/vol) sucrose gradients containing 80% formamide as described in the legend to Fig. 2.

Hybridization conditions. Solution hybridization between RNA, labeled in isolated nuclei, and either Ad2 or restriction endonuclease fragments of ^{32}P-labeled Ad2 DNA prepared as described previously (15) was performed in 0.10 M PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] buffer (pH 6.4) containing 80% formamide and 0.4 M NaCl (7) at 59.5 to 61°C. In experiments to locate viral RNA species among gradient fractions and in saturation hybridization experiments, the hybridization mixture was diluted 10-fold with cold 2× SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) and digested with 10 μg of RNase A, 1 U of RNase T_1, and 1 U of RNase U_2 per ml. Material resistant to RNase digestion was precipitated with 10% trichloroacetic acid and collected on glass-fiber filters, which were dried and counted in Econofluor. The procedures followed for the S1 assay of Berk and Sharp (4) are described in the legend to Fig. 5.

RESULTS

The population of RNA molecules transcribed in nuclei isolated from HeLa cells 18 to 20 h after Ad2 infection includes sequences complementary to the *r* strand of the Ad2 genome from 15 to 17 units to a point to the right of 97.3 units (Yang and Flint, manuscript in preparation). These viral RNA sequences correspond to those of the major, late transcriptional unit depicted in Fig. 1. The rate of incorporation of labeled ribonucleoside monophosphates into RNA declines after about 45 min of incubation (Yang and Flint, in preparation), and we therefore anticipated that the major product of transcription of adenoviral DNA in such isolated nuclei would be the very large RNA molecule whose synthesis is initiated at 16.4 units in the *r* strand (44) and terminated near the right-hand end (15a), as shown in Fig. 1. However, when RNA, ^{32}P-labeled in nuclei isolated 20 h after Ad2 infection, was analyzed in sucrose gradients containing 80% formamide, at least six major species of adenoviral RNA were detected (Fig. 2A); these range in size from 5 to 6S to approximately 55S. Synthesis of the smallest viral RNA species observed, which sediments slightly faster than 4S marker RNA, is resistant to low concentrations (1 μg/ml) of α-amanitin, but is prevented by an inhibitor concentration of 100 μg/ml (Yang and Flint, in preparation). This viral RNA species, then, must be transcribed by RNA polymerase form III and probably represents the VA-RNAs (34, 42). On the other hand, the larger viral RNA species observed must be the products of transcription of Ad2 DNA by form II RNA polymerase, because their synthesis is inhibited by low concentrations of α-amanitin (19, 28).

The discrete nature of the RNA products and the reproducibility with which we have observed them (in at least 10 different experiments) argue against the possibilities that the viral RNA species intermediate between 5 to 6S and 55S result

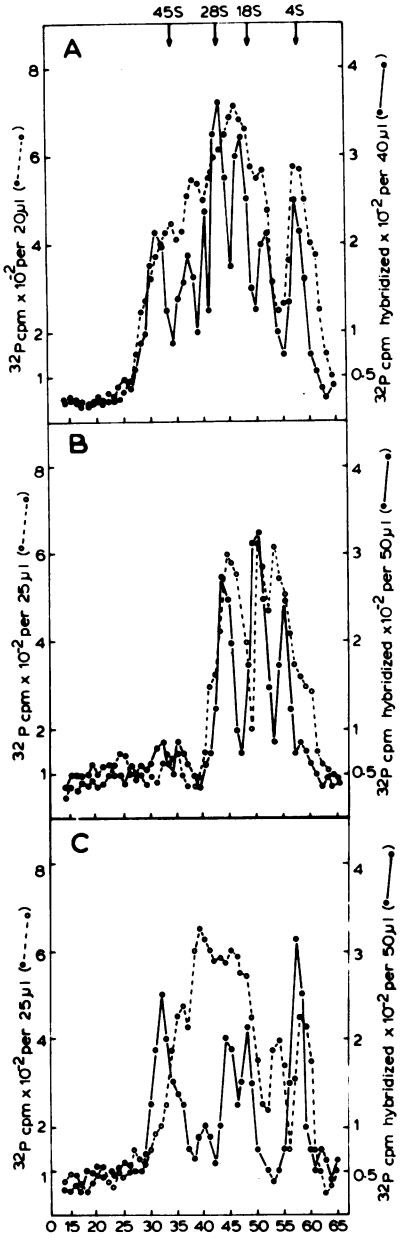

FIG. 2. *Sedimentation of RNA ^{32}P-labeled in isolated nuclei in sucrose gradients containing 80% formamide. RNA was labeled with [α-^{32}P]UTP in nuclei isolated 20 h after Ad2 infection and purified as described in the text. Fractions containing and lacking poly(A) were separated as described in the legend to Table 1. Ethanol-precipitated RNA was collected, denatured, and centrifuged in 15 to 30% sucrose gradients containing 80% formamide, 0.04 M PIPES buffer (pH 7.4), and 1 mM EDTA. Centrifugation was done in a Beckman SW41 rotor at 24°C, 39,000 rpm, for 24 h. Fractions of 0.18 ml were collected from each gradient, and samples were counted directly in Aquasol (New England Nuclear Corp.) (○). Portions of each fraction were also hybridized with 1 µg of Ad2 DNA, and the amount of hybridizing RNA was determined as described in the text (●). In all experiments, marker ^{3}H-labeled nucleolar and ribosomal RNA preparations were centrifuged in parallel gradients. Results were obtained with (A) unfractionated RNA, (B) poly(A)-containing RNA, and (C) poly(A)-lacking RNA.*

from degradation during RNA isolation or random premature chain termination in vitro. Thus it seemed possible that isolated nuclei retain the ability to perform posttranscriptional processing events. We have, therefore, investigated their capacity both to add polyadenylic acid [poly(A)] to late adenoviral RNA sequences transcribed in vitro and to perform the splicing events that generate mature, late adenoviral mRNA species (3, 8, 12, 21).

Polyadenylation of RNA transcribed in vitro. To investigate whether any of the RNA sequences made in nuclei isolated at late times during Ad2 infection were polyadenylated, RNA ^{32}P-labeled in vitro was subjected to chromatography on oligodeoxythymidylic acid [oligo(dT)]-cellulose. Under conditions in which no nucleolar, but 100% of newly made late, adenoviral mRNA is bound to oligo(dT)-cellulose (2), about 30% of the in vitro-labeled RNA is selected as poly(A) containing (Table 1). When such poly(A)-containing RNA is hybridized with saturating amounts of unlabeled Ad2 DNA, some 30% of the RNA enters hybrid (Table 1). These estimates of the fraction of the RNA made in vitro that contains poly(A) (2, 29) and the fraction of polyadenylated RNA that is adenovirus specific (2) are close to those obtained with nuclear RNA newly synthesized in the cell. Sedimentation analysis of the viral RNA species present in the separated poly(A)-containing and -lacking fractions reveals that the two populations are quite different (Fig. 2B and C). It is, for example, clear that 5-6S viral RNA is found exclusively in the fraction lacking poly(A), as would be expected were it VA-RNA. Most of the 55S RNA is also recovered in the fraction lacking poly(A) (see Fig. 2C), whereas the major poly(A)-containing RNA species sediment as molecules of 28S or smaller (in fact, very similar in size to late mRNA species complementary to the *r* strand of Ad2 DNA [16, 25, 26]). This difference between the two populations of in vitro-labeled RNA suggests that polyadenylation of viral RNA made in isolated nuclei does not occur at random sites; the mRNA-like pattern of the polyadenylated, adenoviral RNA species is also consistent with this notion. The spec-

TABLE 1. *Analysis of RNA labeled in nuclei isolated 20 h after Ad2 infection for poly(A)-containing sequences and adenoviral RNA sequences*

Expt	[^{32}P]UMP incorporated[a] (pmol)	% labeled RNA bound to oligo(dT)-cellulose[b]	% Poly(A)-containing RNA hybridized to Ad2 DNA[c]
1	40	32.0	33.3
2	36	28.9	31.2

[a] Nuclei isolated from 6×10^7 infected HeLa cells were incubated under the conditions described in the text in the presence of 200 μCi of [α-^{32}P]UTP.

[b] RNA extracted from isolated nuclei was subjected to two cycles of chromatography on oligo(dT)-cellulose as described elsewhere (12).

[c] These data are taken from saturation hybridization curves in which the poly(A)-containing labeled RNA was hybridized with increasing amounts of Ad2 DNA under the conditions described in the text.

ificity of polyadenylation in vitro has been investigated in greater detail, taking advantage of the fact that poly(A) is added to late nuclear RNA sequences at only the five sites whose locations are shown in Fig. 1 (16, 25, 26).

Poly(A)-containing RNA, into which [α-^{32}P]UMP had been incorporated in nuclei isolated 20 h after Ad2 infection, was selected on oligo(dT)-cellulose columns and subjected to mild alkaline hydrolysis (39) to generate RNA chains of 200 to 400 nucleotides in length. A second cycle of chromatography on oligo(dT)-cellulose was performed to reselect short RNA pieces that contain poly(A). This RNA population, which contains only those sequences adjacent to the sites of poly(A) addition, was then hybridized to restriction endonuclease fragments of Ad2 DNA generated by digestion with *Hin*dIII and *Kpn*I immobilized on nitrocellulose filters (35). Figure 3 shows the result obtained with *Kpn*I digests of Ad2 DNA. Unfractionated RNA labeled in vitro hybridizes to all *Kpn*I fragments A to I, except *Kpn*I fragment G, which comprises the region 0 to 6.5 units in Ad2 DNA (see Fig. 3). On the other hand, fragmented, poly(A)-containing RNA segments hybridize to *Kpn*I fragments A, C, D, and E, which constitute the regions 71.4 to 93.5 units, 23.5 to 39.6 units, 47.4 to 61.3 units, and 61.7 to 71.4 units, respectively, in the Ad2 genome. Thus, these fragments contain the five sites of poly(A) addition depicted in Fig. 1. Similarly, hybridization of this RNA fraction is restricted to *Hin*dIII fragments A (50.1 to 73.6 units), D (41.0 to 50.1 units), F (89.5 to 97.3 units), I (31.5 to 37.3, units), and L (79.9 to 81.0 units) (data not shown)—again those fragments predicted from the scheme of polyadenylation depicted in Fig. 1. From these two sets of data, the sequences adjacent to poly(A) can be located within the regions 31.5 to 37.3 units, 47.4 to 50.1 units, 61.7 to 71.4 units, 79.9 to 81.0 units, and 89.3 to 93.5 units. These results, then, provide strong circumstantial evidence that, in isolated nuclei, poly(A) is added to the five sites in the late precursor RNA shown in Fig. 1. However, as seen from Fig. 3, hybridization of fragmented and poly(A)-selected RNA to *Kpn*I fragment C is highly efficient as compared to that of the other fragments. This seems to indicate that the nuclear system may favor polyadenylation of the mRNA's that have their 3′ ends closest to the promotor.

Splicing of RNA transcribed in vitro. It is clear from the model of synthesis of late adenoviral mRNA depicted in Fig. 1 that, in the absence of additional processing events, the smallest poly(A)-containing RNA generated would extend from 16.4 to near 38 units in the *r* strand, some 8 kilobases in length. However, as shown in Fig. 2B, the population of polyadenylated viral RNA molecules transcribed in vitro includes species as small as 11-13S. The inference from these observations, that RNA transcribed in vitro is processed by mechanisms additional to polyadenylation in isolated nuclei, has been tested by two complementary approaches.

The first of these relies upon the separation of RNA chains elongated or completely synthesized in vitro from those present in the nuclei at the time of cell harvesting through the incorporation of mercurated analogs of ribonucleoside triphosphates, Hg-UTP or Hg-CTP (10, 11). Partial substitution of Hg-CTP for CTP up to ratios of 1:3 has only minimal effects on the properties of, requirements for, and optimal transcriptional activity in nuclei isolated at late times during productive infection (Yang and Flint, submitted for publication). As it has been reported that significant aggregation of mercurated and nonmercurated RNA can occur under certain conditions (22), we have tested various procedures by which mercurated RNA molecules can be selected by chromatography on sulfhydryl agarose columns and developed one that permits the complete separation of RNA chains mercurated in vitro from endogenous nucleic acids (Yang and Flint, submitted for publication). This involves the use of high concentrations of dimethyl sulfoxide during chromatography on sulfhydryl agarose (22).

Once the RNA transcribed in isolated nuclei can be separated from that present in infected cells at the moment of harvesting, it becomes possible to employ the nuclease S1 assay (4) to

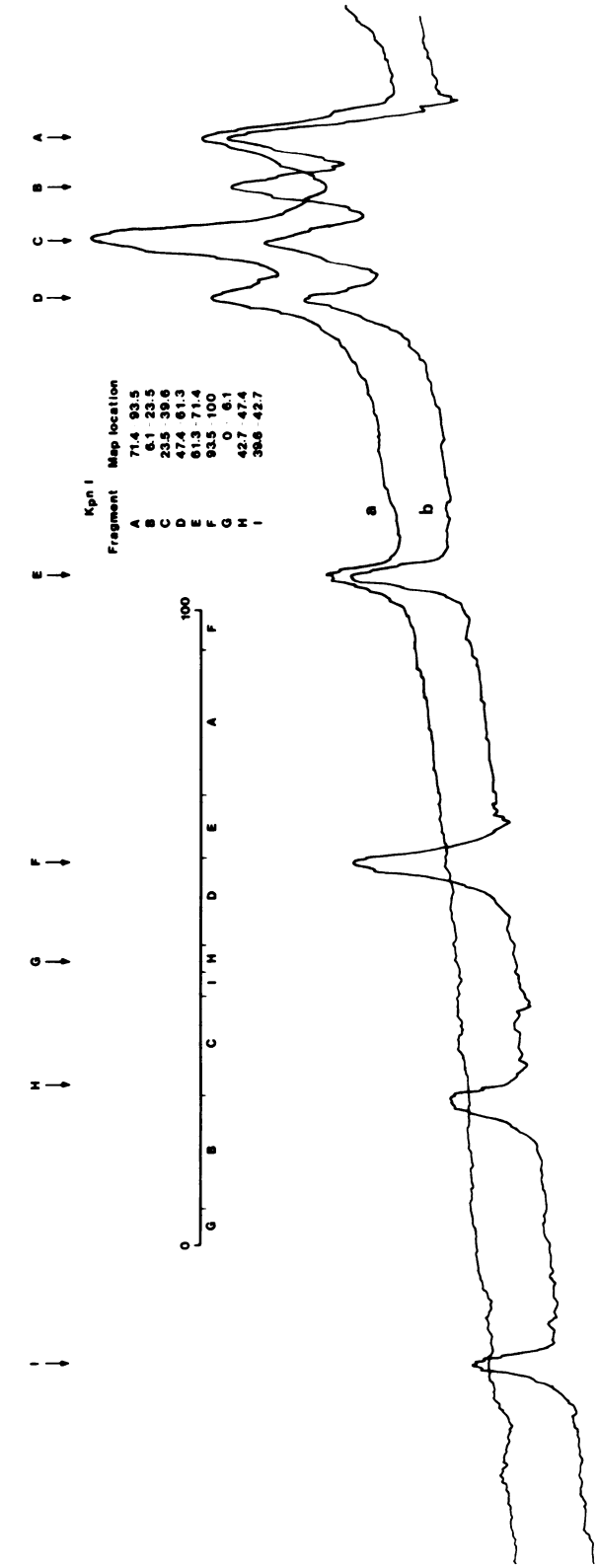

FIG. 3. *Hybridization of unfractionated RNA and fragmented, poly(A)-containing RNA to KpnI fragments of Ad2 DNA. RNA was synthesized in nuclei isolated 20 h after Ad2 infection in the presence of [α-^{32}P]UTP and purified as described in the text. Poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose as described in Table 1 and fragmented in 0.1 M Na_2CO_3 (pH 11.0) (39), and the resulting short poly(A)-containing fragments were reselected by chromatography on oligo(dT)-cellulose. This RNA fraction (a) and unfractionated RNA labeled in vitro (b) were hybridized to strips of nitrocellulose to which 1 μg of Ad2 DNA digested with KpnI had been transferred by the technique of Southern (35). Hybridization was for 20 h at 68°C; this and the subsequent washing steps were performed essentially as described by Botchan et al. (6). The dried filters were mounted on Whatman 3MM paper and exposed to Kodak RP-X-Omat film with an Ilford fast-tungstate intensifying screen. Shown here are tracings of the autoradiograms made with a Joyce-Loebl densitometer.*

determine whether adenoviral RNA molecules transcribed in vitro are spliced. Mercurated RNA, transcribed in nuclei isolated 20 h after Ad2 infection and selected under the stringent conditions described in the previous paragraph, was therefore hybridized to *Bam*HI fragment B, 0 to 29.0 units, of ^{32}P-labeled Ad2 DNA under conditions that permit only DNA:RNA annealing (7). The resulting hybrids were digested with nuclease S1, and the S1-resistant, labeled DNA was subjected to electrophoresis in alkaline agarose gels (4, 24). Spliced RNA molecules can be diagnosed by the presence of a discrete series of labeled DNA bands after autoradiography (4). When this assay was applied to late nuclear RNA isolated directly from Ad2-infected HeLa cells, the pattern shown in Fig. 4, track c, was observed; the largest DNA band seen, about 13.0 units in length, corresponds to that expected from hybridization of ^{32}P-labeled *Bam*HI fragment B to an unspliced RNA molecule extending from 16.4 to the end of the DNA probe, 29.0 units. The single-stranded DNA products of this reaction also include the fragments of approximately 10 units and 3 units in length predicted from the model of synthesis of the segmented leader sequence common to the major late adenoviral mRNA species depicted in Fig. 1. However, a whole host of additional products, ranging in size from less than 1 unit to 11.5 units in length, are also seen (Fig. 4, track c). Similar products have been described by Berget and Sharp (3a) and may be intermediates in the splicing process (3a) or possibly prematurely terminated RNA molecules (13). Be that as it may, it is clear that the pattern observed with late nuclear RNA is quite distinct from that observed when late cytoplasmic RNA was assayed in similar fashion (Fig. 4, track d), but is essentially identical to that obtained with mercurated RNA made in nuclei isolated 20 h after Ad2 infection (Fig. 4, track b). The result shown in Fig. 4, track b, has been observed with several different preparations of mercurated RNA. In addition, when probes such as *Hin*dIII fragment A, 50.1 to 73.6 units, and *Bam*HI fragment C, 42.0 to 59.5 units, were hybridized with mercurated RNA made in vitro, the DNA fragments resolved in alkaline agarose gels were those expected if leader-to-body splicing had occurred in vitro (Yang and Flint, in preparation). Thus, these results suggest that the viral RNA sequences into which Hg-CMP is incorporated in isolated nuclei are indeed spliced to generate a set of RNA products indistinguishable from those made in the Ad2-infected cell.

Although these observations can be taken to indicate that nuclei isolated from adenovirus-infected cells can perform splicing reactions in vitro, it remains possible that the splicing in fact occurred in the cell with nascent viral RNA chains as substrates. These spliced molecules would then be isolated after completion of transcription in vitro in the presence of Hg-CTP. Although this scenario seems unlikely (27, 44), the possibility can be addressed directly by an analysis of the RNA sequences labeled during incubation in vitro. The largest viral RNA molecules made should contain all sequences complementary to the *r* strand of the Ad2 genome from 16.4 units to the extreme right-hand end; any spliced RNA molecule, however, would lack sequences between about 16.45 and 19.5 units and about 19.6 to 26.8 units (see Fig. 1). This prediction was tested by hybridizing RNA species of various sizes, labeled in isolated nuclei and fractionated in gradients like those shown in Fig. 2, to unlabeled fragments of Ad2 DNA generated by cleavage with *Xho*I transferred to nitrocellulose filters (35). The smallest DNA fragment generated by *Xho*I digestion comprises the region from 22.0 to 26.5 units in Ad2 DNA (M. H. Binger, J. Städler, and S. J. Flint, unpublished data) and is therefore a suitable probe with which to assay for splicing at the 5′ ends of viral RNA molecules transcribed in vitro. As can be seen in Fig. 5, only the largest RNA species, about 55S, contains sequences that hybridize to the 22.0 to 26.5-unit *Xho*I fragment of Ad2 DNA; none of the smaller viral RNA species contains detectable sequences complementary to this probe. Because the label was incorporated during transcription in vitro, this result confirms that the late RNA sequences made can also be spliced in isolated nuclei. Moreover, the identity between the splicing patterns observed with late adenoviral RNA made in the intact cell and in isolated nuclei (Fig. 4) indicates that the in vitro splicing reaction is a faithful rendition of the events that occur in the intact cell.

DISCUSSION

It has been known for some time that form II RNA polymerase will transcribe adenoviral DNA in nuclei isolated from productively infected human cells (30, 38), with from 18 to 30% of the in vitro RNA product being virus specific (30, 37, 38, 41). Such adenoviral RNA sequences have also been described to be heterogeneous in size (30, 37) and to be transcribed from the major, *r*-strand transcriptional unit shown in Fig. 1 with characteristics similar to those observed for RNA pulse-labeled in the cell (37, 40, 41). The results presented in Table 1, Fig. 2, and our detailed hybridization analysis of late RNA made in isolated nuclei (Yang and Flint, in prep-

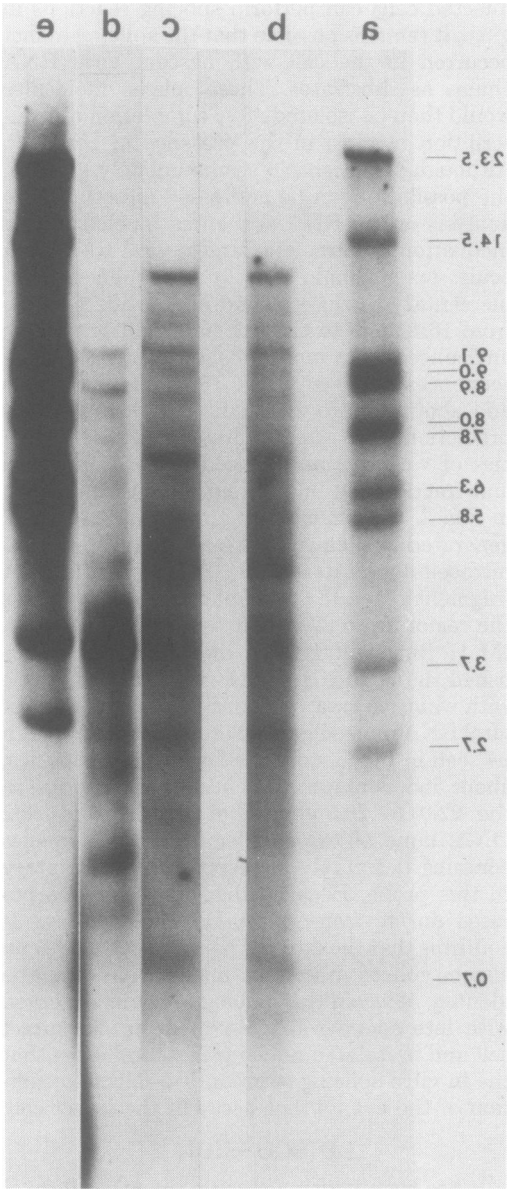

FIG. 4. *Alkaline gel electrophoresis of nuclease S1-digested hybrids formed between ^{32}P-labeled BamHI fragment B and late adenoviral RNA made in vivo and in vitro. Mercurated RNA, synthesized in nuclei isolated 20 h after Ad2 infection and selected as described in the text, was hybridized with ^{32}P-labeled DNA of BamHI fragment B, 0 to 29.0 units of Ad2, after demercuration by incubation in 0.15 M β-mercaptoethanol at 0°C for 8 h (Yang and Flint, in preparation). This hybridization, and those using nuclear and cytoplasmic, poly(A)-containing RNA isolated directly from infected cells 20 h after infection, were performed under the conditions described by Casey and Davidson (7) at 59.5°C. After hybridization for 12 h, samples were diluted 10-fold with 0.03 M sodium acetate (pH 4.3) containing 1 mM $ZnSO_4$ and 5% glycerol and digested with 2 to 10 U of nuclease S1 (Sigma). After ethanol precipitation, the samples were analyzed in 1.4% agarose gels cast in 0.03 M NaCl-1 mM EDTA which were electrophoresed in 0.03 M NaOH-1 mM EDTA. After electrophoresis, gels were dried and autoradiographed as described in the legend to Fig. 3. Molecular weight markers of ^{32}P-labeled Ad2 DNA digested with HindIII are shown in tracks a and e. Tracks b, c, and d show the results of hybridization with (b) RNA synthesized in vitro as described, (c) late nuclear RNA, and (d) late, poly(A)-containing, cytoplasmic RNA.*

aration) are in agreement with these earlier reports. However, by contrast to some other workers (37, 40), we have analyzed the adenoviral RNA species labeled during relatively long periods of incubation in vitro and, as shown in Fig. 2A, find a discrete set of adenoviral RNA species, an observation that led us to question whether RNA sequences made in vitro were subject to processing.

The data presented in Table 1 show that about 30% of the RNA ^{32}P-labeled in vitro is recovered by chromatography on oligo(dT)-cellulose columns under the conditions described. This value is somewhat higher than that reported by Vennström and Philipson (37), but this may reflect the different labeling conditions we used. The results presented in Fig. 3 and obtained from similar experiments discussed in the text provide strong support for the notion that polyadenylation of the RNA made in vitro occurs at the same five sites recognized in nascent RNA in the cell. Direct sequence analysis of the in vitro RNA lying immediately adjacent to poly(A) chains is underway to prove this point conclusively. In view of recent evidence that polyadenylation of late adenoviral RNA takes place before transcription of the unit depicted in Fig. 1 is complete (27), that is, that nascent RNA chains are the substrates for polyadenylation, it is not too surprising that this activity is retained in isolated nuclei. What is more striking is the correspondence between the sizes of the polyadenylated in vitro RNA products shown in Fig. 2B and those reported for late mRNA species (see Fig. 1). This size distribution and the absence from the poly(A)-containing fraction of molecules of the sizes predicted for intermediates in which polyadenylation but no 5′ end processing or splicing had occurred (Fig. 2B) suggested that splicing might indeed occur in isolated nuclei. A recent observation made by Zimmer et al. (45), that discrete sizes of RNA can be synthesized in isolated nuclei, supports this notion.

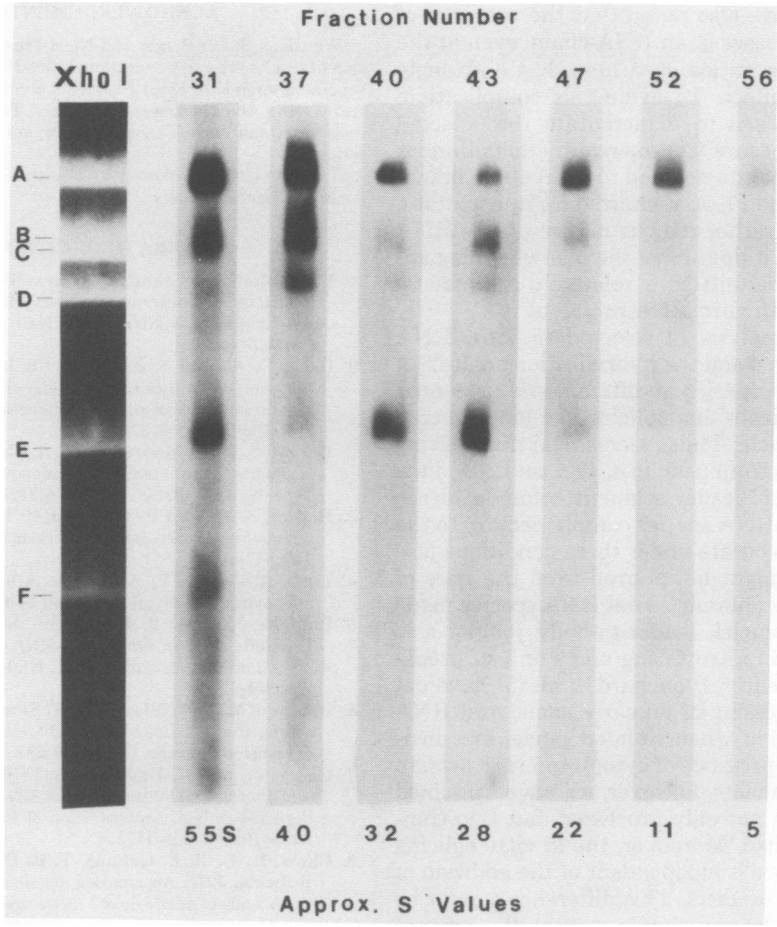

FIG. 5. *Hybridization of fractionated, ^{32}P-labeled RNA synthesized in isolated nuclei to XhoI fragments of Ad2 DNA. RNA was synthesized, in nuclei isolated 20 h after Ad2 infection, in the presence of ^{32}P-labeled UTP and fractioned in denaturing sucrose gradients as described in the legend to Fig. 2. Samples of each fraction were then hybridized to Ad2 DNA as described in the same legend to locate the major species of viral RNA. Appropriate gradient fractions, corresponding to the RNA species whose approximate S values are indicated in the figure, were pooled, and the RNA was hybridized to strips of nitrocellulose to which 1 μg of Ad2 DNA digested with XhoI had been transferred (35). Hybridizations were performed in sealed freezer bags (Sears, Roebuck and Co.) at 68°C under the conditions described by Botchan et al. (6). Washing, processing of the nitrocellulose filters, and autoradiography were performed as described in the legend to Fig. 3. At the left of the figure is shown a photograph of the 1.0% agarose gel in which the XhoI fragments were resolved, stained with ethidium bromide (33).*

To investigate possible splicing steps in isolated nuclei and their fidelity, we have taken advantage of the separation of RNA made in vitro from that present in nuclei at their time of isolation through the incorporation of mercurated ribonucleoside triphosphates (10, 11). It must be pointed out, however, that several obstacles have to be surmounted if the results obtained by this approach are to be interpreted correctly. Although the reactions we have performed in the presence of Hg-CTP in vitro do not appear to suffer from the premature chain termination described by Schäfer (31), perhaps because of our use of relatively low amounts of mercurated substrate analogs, it is clear that extreme care must be taken to achieve complete partition of nonmercurated and mercurated RNA. As Konkel and Ingram (22) reported previously, we find that it is essential to denature the RNA and use stringent washing procedures to ensure such a separation. All RNA preparations we used were, therefore, isolated under the stringent conditions described in detail elsewhere (Yang and Flint, submitted for publica-

tion). We have also noted that the presence of mercurated bases in an RNA chain, even at the relatively low ratios used here, has a strongly denaturing effect. To avoid this complication, we have chosen to demercurate the selected RNA by exposure to β-mercaptoethanol under conditions that do not lead to RNA chain breakage (Yang and Flint, submitted for publication). Despite the rather tedious nature of this RNA preparation, it does have the overwhelming advantage of permitting a relatively fine assessment of the in vitro RNA products.

The S1 analysis of selected in vitro RNA shown in Fig. 4 and the hybridization analysis of labeled RNA species of different size presented in Fig. 5 indicate that splicing can indeed occur in isolated nuclei. It also seems clear that splicing of sequences from near 16.4, 19.6, and 26.8 units to form the 5′ leader segment common to the major late mRNA species complementary to the *r* strand is accurate under these conditions (see Fig. 4). As might be inferred from the sizes of the poly(A)-containing viral RNA species made in isolated nuclei, leader-to-body joining also takes place in vitro (Yang and Flint, in preparation). Recently, Blanchard et al. (5) have described processing of an early adenoviral RNA labeled in vivo when isolated nuclei are incubated in the presence of cytoplasmic extracts. In these experiments, however, we have examined RNA that is not only processed, but also transcribed in vitro. Moreover, the in vitro splicing described here is independent of the addition of cytoplasmic extracts. This difference cannot be rationalized in any simple way at the present time; the method of isolation of nuclei used here is, for example, very similar to that employed by Blanchard et al. (5). On the other hand, it should be pointed out that our understanding of the requirements of splicing enzymes present in eucaryotic cells is poor. In this context, it may be significant that nuclei subjected to sedimentation through sucrose apparently retain relatively little splicing activity (23); similarly, nuclei isolated by nonaqueous methods are unable to process rRNA and tRNA precursors (17).

In summary, then, we have demonstrated that nuclei isolated during the late phase of productive adenovirus infection of human cells retain active and faithful splicing systems. This activity can be detected under conditions of incubation optimized for transcriptional activity, but its explicit requirements have not been determined. Preliminary experiments suggest that at least some activity is retained when nuclei are disrupted (S. J. Flint and S. M. Berget, unpublished data), providing some encouragement towards the goal of purifying the mammalian enzyme(s) that splices mRNA precursors.

ACKNOWLEDGMENTS

We thank Susan Berget and Nigel Fraser for communication of results prior to publication, Mary-Helen Binger for the separated strands of *Sma*I fragments B and D of ^{32}P-labeled Ad2 DNA, Ada Suckewer and Betty Tsang for technical assistance, and Judy Tananis for preparation of the manuscript.

This work was supported by a grant (NP-239) from the American Cancer Society.

LITERATURE CITED

1. **Bachenheimer, S., and J. E. Darnell.** 1975. Adenovirus-2 mRNA is transcribed as part of a high-molecular weight precursor RNA. Proc. Natl. Acad. Sci. U.S.A. **72**:4445–4449.
2. **Beltz, G. A., and S. J. Flint.** 1979. Inhibition of HeLa cell protein synthesis during adenovirus infection: restriction of cellular mRNA sequences to the nucleus. J. Mol. Biol. **131**:353–373.
3. **Berget, S. M., C. Moore, and P. A. Sharp.** 1977. Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. Proc. Natl. Acad. Sci. U.S.A. **74**:3171–3175.

3a. **Berget, S. M., and P. A. Sharp.** 1979. Structure of late adenovirus 2 heterogeneous nuclear RNA. J. Mol. Biol. **129**:547–565.

4. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14**:695–711.
5. **Blanchard, J. M., J. Weber, W. Jelinek, and J. E. Darnell.** 1978. *In vitro* RNA-RNA splicing in adenovirus 2 mRNA formation. Proc. Natl. Acad. Sci. U.S.A. **75**:5344–5348.
6. **Botchan, M., T. William, and T. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
7. **Casey, J., and N. Davidson.** 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentration of formamide. Nucleic Acids Res. **4**:1539–1552.
8. **Chow, L. T., R. E. Gelinas, T. R. Broker, and R. J. Roberts.** 1977. An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. Cell **12**:1–8.
9. **Chow, L. T., J. M. Roberts, J. B. Lewis, and T. R. Broker.** 1977. A map of cytoplasmic RNA transcripts from lytic adenovirus type 2, determined by electron microscopy of RNA:DNA hybrids. Cell **11**:819–836.
10. **Dale, R. M. K., E. Martin, D. C. Livingston, and D. C. Ward.** 1975. Direct covalent mercuration of nucleotides and polynucleotides. Biochemistry **14**:2447–2457.
11. **Dale, R. M. K., and D. C. Ward.** 1975. Mercurated polynucleotides: new probes for hybridization and selective polymer fractionation. Biochemistry **14**:2458–2469.
12. **Dunn, A. R., and J. A. Hassell.** 1977. A novel method to map transcripts: evidence for homology between an adenovirus mRNA and discrete multiple regions of the viral genome. Cell **12**:23–36.
13. **Evans, R., J. Weber, E. Ziff, and J. E. Darnell.** 1979. Premature termination during adenovirus transcription. Nature (London) **278**:367–370.
14. **Flint, S. J.** 1977. The topography and transcription of the adenovirus genome. Cell **10**:153–166.
15. **Flint, S. J., P. H. Gallimore, and P. A. Sharp.** 1975. Comparison of viral RNA sequences in adenovirus 2 transformed and lytically infected cells. J. Mol. Biol. **96**:47–68.

15a. **Fraser, N., J. R. Nevins, E. Ziff, and J. E. Darnell.** 1979. The major late Ad2 transcription unit: termination is downstream from the last poly(A) site. J. Mol. Biol. **129**:643–656.

16. **Fraser, N., and E. Ziff.** 1978. RNA structures near poly(A) of adenovirus 2 late messenger RNAs. J. Mol.

Biol. 124:27-51.
17. **Gurney, T. J.** 1978. Use of isolated nuclei to synthesize and stabilize initial RNA transcripts. J. Cell Biol. **79**: 349a.
18. **Jaehning, J. A., and R. G. Roeder.** 1977. Transcription of specific adenovirus genes in isolated nuclei by exogenous RNA polymerases. J. Biol. Chem. **252**:8753-8761.
19. **Kedinger, G., M. Ginazdowski, J. L. Mendel, F. Gissinger, and P. Chambon.** 1970. α-Amanitin: a specific inhibitor of one of two DNA-dependent RNA polymerase activities from calf thymus. Biochem. Biophys. Res. Commun. **38**:165-171.
20. **Kitchingham, G. R., S. P. Lai, and H. Westphal.** 1977. Loop structures in hybrids of early RNA and the separated strands of adenovirus DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:4392-4395.
21. **Klessig, D. F.** 1977. Two adenovirus mRNAs have a common 5' terminal leader sequence encoded at least 10 kb upstream from their main coding regions. Cell **12**: 9-21.
22. **Konkel, D. A., and V. M. Ingram.** 1977. RNA aggregation during sulfhydryl agarose chromatography of mercurated RNA. Nucleic Acids Res. **4**:1979-1988.
23. **Manley, J., P. A. Sharp, and M. L. Gefter.** 1979. RNA synthesis in isolated nuclei: *in vitro* initiation of adenovirus 2 major late mRNA precursor. Proc. Natl. Acad. Sci. U.S.A. **76**:160-164.
24. **McDonell, 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. **110**:119-146.
25. **McGrogan, M., and H. J. Raskas.** 1978. Two regions of the adenovirus 2 genome specify families of late polysomal RNAs containing common sequences. Proc. Natl. Acad. Sci. U.S.A. **75**:625-629.
26. **Nevins, J. R., and J. E. Darnell.** 1978. Groups of adenovirus type 2 mRNAs derived from a large primary transcript: probable nuclear origin and possible common 3' ends. J. Virol. **25**:811-823.
27. **Nevins, J. R., and J. E. Darnell.** 1978. Steps in the processing of Ad2 mRNA: poly(A)$^+$ and nuclear sequences are conserved and poly(A) addition precedes splicing. Cell **15**:1477-1493.
28. **Novello, F., and F. Stirpe.** 1970. Simultaneous assay of RNA polymerases I and II in nuclei isolated from resting and growing rat liver with the use of α-amanitin. FEBS Lett. **8**:57-60.
29. **Philipson, L., U. Pettersson, U. Lindberg, C. Tibbetts, B. Vennström, and T. Persson.** 1975. RNA synthesis and processing in adenovirus infected cells. Cold Spring Harbor Symp. Quant. Biol. **39**:447-456.
30. **Price, R., and S. Penman.** 1972. Transcription of the adenovirus genome by an α-amanitine-sensitive ribonucleic acid polymerase in HeLa cells. J. Virol. **9**:621-626.
31. **Schäfer, K. P.** 1977. Mercurated nucleotides: assessment of a new tool to study RNA synthesis and processing in isolated nuclei. Nucleic Acids Res. **4**:4465-4479.
32. **Seeburg, P. H., J. Shine, J. A. Martial, A. Ullrich, J. D. Baxter, and H. M. Goodman.** 1977. Nucleotide sequence of part of the gene for human chorionic somatomammotropin: purification of DNA complementary to predominant mRNA species. Cell **12**:157-165.
33. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry **12**: 3055-3063.
34. **Söderlund, H., U. Pettersson, B. Vennström, L. Philipson, and M. B. Mathews.** 1976. A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. Cell **7**:585-593.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.
36. **Vennström, B., V. Pettersson, and L. Philipson.** 1978. Two initiation sites for adenovirus 5.5S RNA. Nucleic Acids Res. **5**:195-204.
37. **Vennström, B., and L. Philipson.** 1977. Fidelity of adenovirus RNA transcription in isolated HeLa cell nuclei. J. Virol. **22**:290-299.
38. **Wallace, R. D., and J. Kates.** 1972. State of adenovirus 2 deoxyribonucleic acid in the nucleus and its mode of transcription: studies with isolated viral deoxyribonucleic acid-protein complexes and isolated nuclei. J. Virol. **9**:627-635.
39. **Wang, L.-H., P. Duesberg, K. Beemon, and P. K. Vogt.** 1975. Mapping RNase T_1-resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation-defective viruses are at the poly(A) end. J. Virol. **16**:1051-1070.
40. **Weber, J., W. Jelinek, and J. E. Darnell.** 1977. The definition of a large viral transcription unit late in Ad2 infection of HeLa cells. Mapping of nascent RNA molecules labeled in isolated nuclei. Cell **10**:611-616.
41. **Weinmann, R., and L. O. Aiello.** 1978. Mapping of adenovirus late promoters with nascent mercurated RNA. Proc. Natl. Acad. Sci. U.S.A. **75**:1662-1666.
42. **Weinmann, R., J. A. Jaehning, H. J. Raskas, and R. G. Roeder.** 1976. Viral RNA synthesis and levels of DNA-dependent RNA polymerase during replication of adenovirus 2. J. Virol. **17**:114-126.
43. **Williams, J. F.** 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. **9**:251-255.
44. **Ziff, E., and R. Evans.** 1978. Coincidence of the promoter and capped 5' terminus of RNA from the adenovirus 2 major late transcription unit. Cell **15**:1463-1475.
45. **Zimmer, S. G., C. J. Goldenberg, D. P. Carlson, E. A. Craig, and H. J. Raskas.** 1978. Size distribution of polyadenylated adenovirus 2 RNA synthesized in isolated nuclei. Biochemistry **17**:4207-4213.