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Size Distribution of Polyadenylated Adenovirus 2 RNA Synthesized in Isolated Nuclei†

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ABSTRACT: Viral RNA synthesis was studied in nuclei isolated 14 h after adenovirus 2 infection of KB cells. RNA synthesized in vitro was approximately 40% viral and was derived almost exclusively from the *r* strand (95%). Approximately 30% of the nucleotides incorporated in vitro bound to oligo(dT)-cellulose. These values are comparable to those obtained for RNA synthesized in vivo. Hybridization-inhibition experiments demonstrated that the in vitro transcripts include two classes of sequences, those restricted to the nucleus in vivo and those present in cytoplasmic RNA. Quantitation of viral sequences from defined regions of the genome was accomplished by exhaustive hybridization. Hybridization was performed with seven viral DNA fragments generated by digestion with either endo R·*Eco*R1 or endo R·*Sma*I. Of the regions tested, the most abundant RNAs were derived from genome positions 39–52 and 77–91. Some regions of the genome were transcribed in eightfold greater amounts per unit length than other regions. Similar results were obtained with RNAs labeled in vitro and in vivo. The size distribution of polyadenylated viral RNA in nuclei incubated in vitro was examined by two types of experiments. First, the sizes of viral RNAs synthesized in vitro were determined, and in other experiments size distributions were determined for viral RNAs labeled in vivo and then incubated in vitro. Polyadenylated viral RNAs synthesized in vitro ranged in size from 13S to 28S with peaks of 28S, 25S, and 22S RNAs superimposed on a heterogeneous background. The polyadenylated nuclear RNA labeled during a 30-min in vivo pulse was considerably larger than the in vitro product, varying in size from 23S to 36S. Incubation of the prelabeled nuclei in RNA synthesis conditions resulted in a gradual size change of the prelabeled RNAs to a distribution similar to that of the in vitro products. *Eco*R1 fragments E (83–89) and C (89–100) were used to perform a more detailed analysis of nuclear RNAs transcribed from regions encoding the 22S fiber mRNA. Hybridization with both fragments revealed that discrete 28S, 25S, and 22S RNAs were synthesized in vitro. RNA prelabeled in vivo contained 36S, 28S, and 22S RNAs which were converted during in vitro incubation to a size distribution identical to that of the in vitro products. Such in vitro systems offer the possibility of studying coupled mRNA transcription and processing.

Studies of viral RNA synthesis in human cells productively infected with adenovirus 2 have elucidated differences between the nuclear transcripts and the cytoplasmic mRNAs. The cytoplasmic RNAs synthesized late in infection consist of a set of at least 12 mRNAs which are polyadenylated at the 3′ end and capped at the 5′ terminus (McGrogan and Raskas, 1977; Chow et al., 1977a; Gelinas and Roberts, 1977). In contrast, the nuclear fraction consists of a heterogeneous population of poly A(+) and poly A(−) molecules (Philipson et al., 1971). The nuclear RNAs include molecules significantly larger than the largest cytoplasmic mRNAs (Parsons et al., 1971; Bachenheimer and Darnell, 1975); some molecules may be transcripts of a DNA segment as large as 80% of the 35 000 nucleotide viral genome (Meissner et al., 1977; Weber et al., 1977). In addition, the nuclear RNAs contain sequences that are not present in the cytoplasmic fraction (Thomas and Green, 1969; Philipson et al., 1974; Sharp et al., 1974).

At late times in infection, after the onset of viral DNA synthesis, greater than 95% of the newly synthesized viral mRNA is derived from the viral *r* strand (Pettersson and Philipson, 1974). Several types of experiments have provided evidence that the large nuclear transcripts are precursors of these mRNAs and that a major promotor for rightward transcription is located at approximately map position 20 on the viral genome (Bachenheimer and Darnell, 1975; Weber et al., 1977; Goldberg et al., 1977; Evans et al., 1977). Recent structural studies of the late viral mRNAs have demonstrated the existence of a 150–200 nucleotide sequence common to the late mRNAs transcribed from map positions 20–100 (Chow et al., 1977b; Berget et al., 1977; Klessig, 1977). This leader sequence is located at the 5′ end of the mRNAs and includes sequences from genome positions 17, 20, and 27. This finding provides further evidence that the region around position 20 constitutes the major promotor for late rightward transcription.

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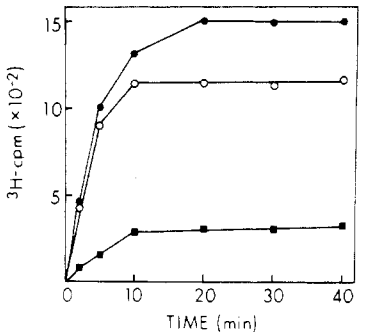

FIGURE 1: Kinetics of RNA synthesis in nuclei isolated 14 h after adenovirus 2 infection. Nuclei isolated as described under Materials and Methods were incubated under conditions optimal for RNA polymerase II activity (see Materials and Methods). Reactions were performed in a volume of 0.5 mL. At the indicated times, incorporation was assayed in 50-μL samples. When α-amanitin was used to inhibit RNA polymerase, it was present at a final concentration of 1.2 μg/mL: (●–●) no α-amanitin; (○–○) α-amanitin added at 10 min; (■–■) α-amanitin added at 0 min.

An in vitro system for studying coupled RNA transcription and processing would be of considerable value in elucidating the precursor–product relationships that govern formation of viral mRNA. We have purified nuclei isolated from cultures late in adenovirus 2 infection and studied their ability to transcribe viral RNA with fidelity and to generate discrete viral RNA size classes from specific regions of the genome.

A preliminary report of part of this data was presented at the 1976 EMBO Workshop on Symmetrical Transcription and Post-Transcriptional Controls.

Materials and Methods

Cell Culture and Virus Infection. Suspension cultures of KB cells were maintained in Joklik modified minimal essential medium supplemented with 5% horse serum. Infections with adenovirus 2 were performed as previously described (Craig et al., 1975) at a multiplicity of 50 pfu/cell. After a 1-h adsorption period at 6–8 × 10^6 cells/mL, the culture was diluted to 3 × 10^5 cells/mL and kept at 37 °C until harvested at 14–15 h postinfection.

Preparation of Nuclei. At 14–15-h postinfection, the cells, usually 500 mL of culture, were rapidly chilled by pouring over frozen phosphate-buffered saline (PBS) and harvested by centrifugation at 800*g* for 10 min. The pelleted cells were then washed with PBS and the resulting cell pellet was resuspended in 10 volumes of isotonic buffer (30 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT).[1] The nuclei were separated from the cytoplasm by Dounce homogenization in the presence of 0.1% NP-40 (Shell Chemical Co.). Nuclei were collected by centrifugation at 800*g* for 10 min. The nuclear pellet was resuspended in 5 mL of isotonic buffer and centrifuged through an equal volume of nuclear synthesis buffer (30 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 150 mM KCl, 20% glycerol). The pelleted nuclei were resuspended in 2× nuclear synthesis buffer at a final concentration of 4 × 10^7 nuclei/mL. Purity of nuclei was monitored by phase contrast microscopy.

Nuclear RNA Synthesis. The final concentration of each component in the reaction mixture was 30 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM each of GTP, CTP, and ATP, 0.01 mM UTP, 10 mM creatine phosphate, 100 μg/mL creatine phosphokinase, 1 mCi/mL [^{3}H]UTP (39 Ci/mmol), and 2 × 10^7 nuclei/mL. The incubation was carried out at 30 °C. For preparation of RNA, the reaction volume was usually 1 mL.

The amount of [^{3}H]UTP incorporated was measured by removing 50-μL samples and mixing with 50 μL of a buffer containing 10 mM unlabeled UTP, 0.3 M KCl, 20 mM EDTA, and 0.5% $NaDodSO_4$. The 100-μL sample was then applied to a 2.4-cm DEAE filter (DE 81, Whatman), washed for 5 min four times with 5% KH_2PO_4, three times with H_2O, two times with 95% ethanol, and two times with ether, dried, and counted by scintillation spectrophotometry (Blatti et al., 1970).

Purification of RNA. RNA synthesized in vitro was extracted with phenol as described by Holmes and Bonner (1973). After incubation at 30 °C, a 1 mL reaction was terminated by the addition of 4 mL of urea solution [7 M urea, 0.35 M urea, 0.35 M NaCl, 10 mM Tris-HCl (pH 8), 20 mM EDTA, 2% $NaDodSO_4$]. The RNA was extracted with an equal volume of phenol [saturated with 0.1 M NaCl, 0.01 M Tris (pH 7.5), and 0.002 M EDTA] and chloroform–isoamyl alcohol (at a ratio of 24:1) until the interphase was clear. The nucleic acids were precipitated with 2.5 volumes of 95% ethanol. The precipitate was dissolved in 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.05 M Tris-HCl (pH 7.4) and digested with 100 μg/mL DNase I (RNase free, Worthington, DPFF). The RNA was extracted with phenol and again precipitated with 2.5 volumes of 95% ethanol. In some instances, standard chloroform–isoamyl alcohol–phenol extractions were performed (Craig and Raskas, 1975) instead of the urea method.

RNA containing poly(adenylic acid) [poly(A)] was selected by chromatography on oligo(deoxythymidylic acid)-cellulose [oligo(dT)-cellulose] (Collaborative Research, Inc.; Aviv and Leder, 1972) and precipitated with 2 volumes of 95% ethanol.

Preparation of DNA Fragments and Nucleic Acid Hybridization. DNA–DNA hybridizations were performed by incubating ^{32}P-labeled DNA to adenovirus 2 DNA fragments which had been immobilized on 6.5-mm nitrocellulose filters (Nishimoto et al., 1975). The ^{32}P-labeled DNA was sonicated to an average size of approximately 300 nucleotides and denatured in alkali (0.1 N NaOH for 10 min in 0.1× SSC). Hybridizations were performed for 24 h at 60 °C in 200 μL of 2× SSC–0.1% $NaDodSO_4$. After incubation, the unhybridized DNA was removed by washing the filters ten times with 3 mM Tris-HCl (pH 9.0).

RNA–DNA hybridizations were performed with DNA immobilized on nitrocellulose filters (6.5 mm) (Craig and Raskas, 1974). Incubation was for 20–24 h in 200 μL of 6× SSC–0.1% $NaDodSO_4$. The filters were washed three times in 2× SSC, treated with 20 μg/mL RNase A for 1 h at room temperature, followed by an additional three washings in 2× SSC. Hybridization-inhibition experiments were performed as described by Craig et al. (1975). DNA fragments were prepared by digestion with either endo R·*Eco*R1 or endo R·*Sma*I as previously described (Craig and Raskas, 1976). Amounts of DNA fragments are given as microgram equivalents, i.e., the amount of the fragment that would be derived from 1 μg of whole genome DNA.

Results

RNA synthesis was studied using nuclei isolated 14 h after infection of KB cells with adenovirus 2. This time after infection has been shown previously to be optimal for synthesis of late viral RNAs (Weinmann et al., 1976).

[1] Abbreviations used: DTT, dithiothreitol; ATP, CTP, GTP, and UTP, adenosine, cytidine, guanosine and uridine 5′-triphosphates; EDTA, ethylendiaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), standard saline citrate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Viral Content and Strand Assignment of RNAs Synthesized in Isolated Nuclei.[a]

	RNA	
	in vitro	in vivo
% viral-specific RNA		
expt 1	57%	25%
expt 2	38%	38%
cpm hybridized to adenovirus 2 *r* strand		
expt 1	905	342
expt 2	882	321
cpm hybridized to adenovirus 2 *l* strand		
expt 1	40	41
expt 2	18	20

[a] The percentage of viral-specific RNA was determined in the following manner: 3000–4000 cpm of RNA labeled in vitro or in vivo was hybridized to nitrocellulose filters containing either 1, 2, or 4 μg of adenovirus 2 DNA. Cpm hybridizing in DNA excess was used to calculate the percentage of viral RNA in the sample. Hybridization to separated adenovirus 2 *r* and *l* strands was performed with inputs of 2000 (in vitro) and 1000 cpm (in vivo); 2 μg of each strand was used. As a control, 4000 cpm of cytoplasmic RNA labeled early in infection in vivo was hybridized to 2 μg of separated *r* or *l* strand DNA. This RNA annealed 65% (320 cpm) to the *l* strand and 35% (175 cpm) to the *r* strand.

Kinetics of RNA Synthesis. Initial studies involved the analysis of the kinetics of in vitro RNA synthesis. Samples were removed at various times after the start of synthesis and assayed for [^{3}H]UTP incorporation. As shown in Figure 1, the reaction generally exhibited plateau kinetics, although in several experiments there was a decrease of 10–20% during prolonged (40 min) incubations. Incorporation was essentially linear for the first 5–10 min and then progressed at a continuously decreasing rate. To determine the relative synthetic activities of RNA polymerases II and III, α-amanitin was added to the reaction in concentrations which inhibit only polymerase II activity (Figure 1). With α-amanitin present, incorporation was inhibited 80%, demonstrating that in these ionic conditions RNA polymerase II is responsible for the major synthetic activity. To examine the possibility that the net incorporation of [^{3}H]UTP represents the sum of synthetic and degradative activities, synthesis was allowed to proceed for 10 min and α-amanitin was then added. As shown in Figure 1, the number of counts incorporated did not significantly decrease from 10 to 40 min, indicating that extensive degradation of the RNA did not occur.

Characterization of in Vitro RNA Products. The fidelity of transcription was determined by comparing the in vitro products to RNAs synthesized in vivo at 14-h postinfection. In vitro RNAs were labeled for 10 min and in vivo molecules for 4 min. Exhaustive hybridization with adenovirus 2 DNA revealed that both in vivo and in vitro RNAs usually contained 30–50% viral sequences (Table I).

Most of the RNA synthesized at late times is derived from the viral *r* strand, the strand transcribed in the rightward direction (Pettersson and Philipson, 1974). Both the in vivo and in vitro nuclear RNAs hybridized approximately 95% to the *r* strand (Table I). As a control, early cytoplasmic RNA was hybridized to the separated *r* and *l* strands. As expected, about two-thirds of the RNA annealed to the viral *l* strand (data not shown). Hybridization–inhibition experiments were performed to exclude the possibility that the in vitro products are derived predominantly from regions that do not code for mRNA.

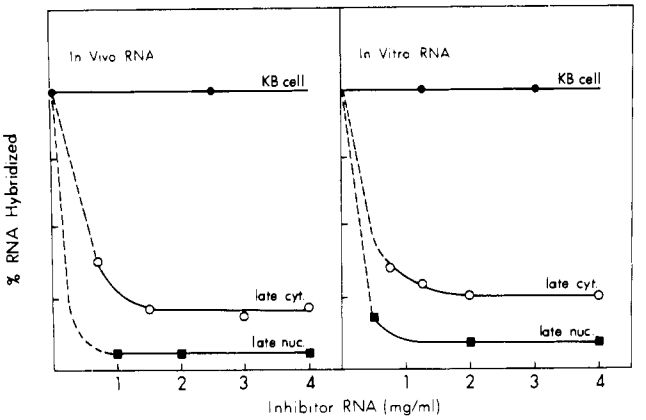

FIGURE 2: Hybridization–inhibition analyses of in vitro and in vivo nuclear RNAs. Hybridization–inhibition experiments were performed either with RNA labeled for 4 (in vivo, left panel) or 10 min (in vitro, right panel). Three types of inhibitor RNAs were used, RNA from uninfected KB cells (●–●), cytoplasmic RNA from cultures harvested 14 h after infection (O- -O), or nuclear RNA from cultures harvested at 14 h (■–■). The value for 100% hybridization with in vivo RNAs was 850 cpm and for the in vitro products was 950 cpm. Hybridization–inhibition procedures were identical to those of Craig et al. (1975a). The amount of DNA was 1 μg/membrane. Radioactive RNAs were used in amounts sufficient to give 80% of the hybridization attained at saturation values.

Hybridizations were performed with either nuclear [^{3}H]RNA from isolated nuclei or with nuclear [^{3}H]RNA labeled in vivo (Figure 2). The hybridization of both in vivo and in vitro [^{3}H]RNAs was inhibited nearly 100% by nuclear RNA but only 75–80% by cytoplasmic preparations. The results confirm that the in vitro transcripts include mRNA and non-mRNA sequences as do the in vivo nuclear RNAs.

Polyadenylation of the RNAs transcribed in vitro was examined by chromatography on oligo(dT)-cellulose (Table II). Nuclear RNA synthesized in vivo during brief labeling periods bound to oligo(dT)-cellulose 10–30%. The binding of the in vitro product was found to be highly variable with normal extraction procedures, ranging from 3 to 30%. This variation was eliminated by utilization of an extraction procedure that includes treatment with urea (Table II). Binding was routinely 25–35%.

Quantitation of Genome Regions Specifying in Vitro Viral Transcripts. The amounts of viral RNA transcribed from different regions of the genome in the in vitro system were compared to the in vivo products. Figure 3 shows the genome segments represented by the DNA fragments chosen for this study. The fragments include endo R·*Eco*R1 B, F, and C and endo R·*Sma*I E, F, D, and C. Quantitation of RNA transcripts by hybridization in DNA excess requires confirmation of the hybridization efficiency of individual fragment filters. Therefore, each filter was pretested by DNA–DNA hybridization to determine the hybridization efficiency under conditions of DNA excess and equivalence (Table III). The relative hybridization capacity of each fragment was determined, normalizing to the DNA having the highest efficiency. The efficiency ranged from 75 to 100%. All filters were also tested for ability to hybridize late cytoplasmic [^{3}H]RNA and were shown to be competent.

The results of exhaustive hybridization of RNA synthesized in vitro are shown in Figure 4. The highest levels of hybridization were found with *Sma*I fragments D and C; intermediate levels were obtained with *Eco*R1 fragments B and C followed closely by *Sma*I fragments E and F. Low levels of hybridization were obtained with *Eco*R1 F fragment. Since the test fragments vary in size (Table III), the data are more readily compared when they are expressed in terms of cpm

TABLE II: Binding of in Vitro RNA to Oligo(dT)-cellulose.[a]

RNA source	extraction procedure	RNA not bound (cpm × 10^{-4})	RNA bound (cpm × 10^{-4})	binding %
in vitro	expt 1			
	non-urea	4.28	0.4	8.5
	urea	4.68	2.1	31
	expt 2			
	non-urea	6.50	0.21	3.1
	urea	4.21	2.7	39
in vivo	urea	627	282	31

[a] Nuclei from 2 × 10^7 cells were purified and incubated with 200 μCi of [^{3}H]UTP in 1 mL of reaction mixture as described under Materials and Methods. At the end of the incubation period (10 min at 30 °C), the reaction was terminated in one of two ways: An equal volume of 20 mM EDTA, 0.5 M KCl, 0.01 M Tris-HCl (pH 7.5), 1% sarcosyl was added to 0.5 mL of the mixture; 2 mL of urea solution [7 M urea, 0.01 M Tris (pH 8), 20 mM EDTA, 0.35 M NaCl, 1% sarcosyl] was added to the remaining half. RNA was purified as described under Materials and Methods and poly(A)-containing molecules were selected by chromatography on oligo(dT)-cellulose. RNAs labeled in vivo were purified from a culture labeled for 30 min beginning 14 h after infection.

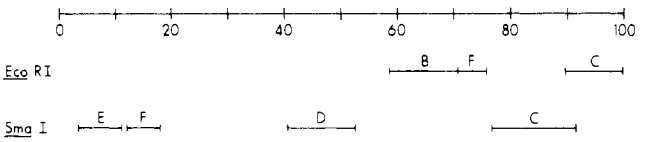

FIGURE 3: Adenovirus 2 DNA fragments used to analyze genome sites coding for late nuclear RNAs. The indicated endo R·*Eco*R1 and endo R·*Sma*I fragments were used in the studies of Figures 4 and 5. Molecular weights of the fragments are given in Table III.

TABLE III: Hybridization Competence of DNA Fragment Filters.[a]

DNA fragment	mol wt (×10^{-6})	rel eff in DNA-DNA hybrid	cpm of RNA hybridized
*Sma*I E	1.86	1.00	548
*Sma*I F	1.52	0.89	724
*Sma*I D	2.78	0.74	2080
*Eco*RIB	2.81	0.94	3248
*Eco*RI F	1.20	0.75	447
*Sma*I C	3.45	0.92	2122
*Eco*R1 C	2.37	0.73	1400

[a] The molecular weights described were determined from the restriction enzyme cleavage studies of Mulder et al. (1974) and McGrogan and Raskas (1978). Hybridization competence of the filters was determined by annealing with viral [^{3}H]DNA in tenfold excess. The cpm hybridizing were arbitrarily normalized to the *Sma*I E membranes. Based on the specific activity of the [^{3}H]DNA, the *Sma*I E membranes hybridized 1.12 μg equiv of DNA in comparison to the 1-μg equiv expected. RNA hybridization utilized polyadenylated [^{3}H]RNA labeled 12–14 h after infection. Input cpm for this hybridization were 60 000.

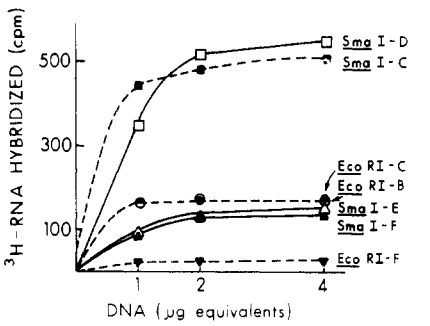

FIGURE 4: Exhaustive hybridization of polyadenylated RNA synthesized in vitro. RNA synthesized during a 10-min incubation was annealed simultaneously to the seven DNA fragments indicated. One set of hybridizations utilized membranes containing 1-μg equiv of the fragments, a second set contained 2-μg equiv, and a third set had 4-μg equiv. The hybridization competence of these fragment filters was tested by DNA-DNA or RNA-DNA hybridization; results for the microgram filters were presented in Table III. Hybridization was performed as described under Materials and Methods. Input RNA was 4000 cpm.

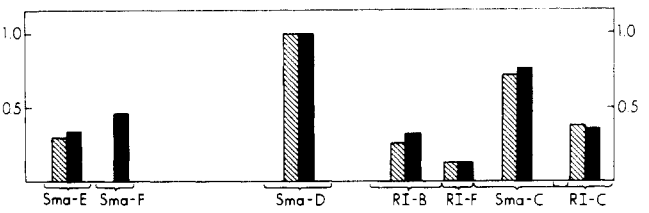

FIGURE 5: Relative amounts of in vivo and in vitro RNAs hybridizing to specific regions of the genome. The results of the exhaustive hybridization studies in Figure 4 and a similar analysis of RNA labeled in vivo for 4 min were normalized to unit length DNA. The genome region specifying the greatest amount of [^{3}H]RNA (*Sma*ID) was assigned a value of 1.0. The solid bars show the results for polyadenylated in vitro RNAs, and the cross-hatched bars are the data from an in vivo preparation.

hybridized per unit genome length. These calculations were performed for each individual fragment and then normalized to *Sma*ID, the fragment with the highest ratio of cpm per unit length DNA. The results of these calculations are summarized in Figure 5. The amount of RNA transcribed from the *Sma*I D fragment was approximately 1.4-fold higher than that derived from *Sma*I C, 2.8-fold higher than *Eco*R1 B, and at least sevenfold higher than that derived from *Eco*R1 F.

The above results were compared to those obtained with nuclear RNAs labeled in vivo at 14 h postinfection. A brief labeling time of 4 min was chosen to minimize the possibility of selective RNA degradation in vivo. The distribution of hybridized [^{3}H]RNA was essentially the same as that obtained with the in vitro transcripts (Figure 5). Since the populations of polyadenylated and nonpolyadenylated RNAs might contain different distributions of transcripts from various genome regions, identical experiments were performed with total nuclear RNA preparations and poly A(−) RNAs labeled both in vivo and in vitro. In all cases, similar distributions were obtained (data not shown).

Size of the in Vitro Polyadenylated RNA. The polyadenylated RNA synthesized in isolated nuclei was analyzed by fractionation in polyacrylamide gels containing 98% formamide followed by hybridization to adenovirus 2 DNA. The RNA was eluted from each gel fraction and annealed to 1 μg of adenovirus 2 DNA (Figure 6; right panel). The in vitro viral

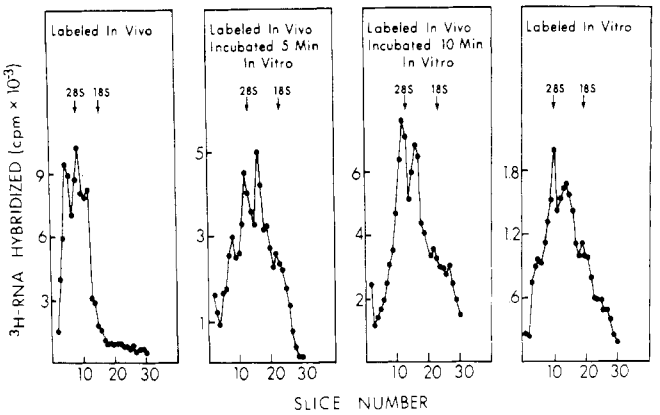

FIGURE 6: Size distribution of polyadenylated viral nuclear RNAs. RNAs were labeled either in vivo (three left panels) or in vitro (right panel). For in vivo labeling, cultures were concentrated to 9×10^5 cells/mL. The RNA was then pulse labeled with [^{3}H]uridine (50 μCi/mL; 40 Ci/mmol) for 30 min. Nuclei were isolated with buffers containing 10 mM nonradioactive uridine and incubated for 0, 5, and 10 min in the standard RNA synthesis mixture at 30 °C, including 0.5 mM nonradioactive UTP. At the indicated times the reaction was stopped, and polyadenylated RNA was purified. Nuclear RNA synthesized in vitro was labeled with [^{3}H]UTP for the first 10 min of the incorporation; the reaction was terminated by the addition of EDTA (20 mM) and sarcosyl (1%). The RNA preparations were denatured and subjected to electrophoresis in 98% formamide gels for 10 h at 2 mA/gel (Duesberg and Vogt, 1973; Craig and Raskas, 1976). The RNA eluted from each gel slice was hybridized to 1 μg of adenovirus 2 DNA. The arrows indicate the positions to which 28S and 18S RNA migrate in these gels. The positions were established by coelectrophoresis of [^{14}C]rRNA and smaller aliquots of the RNA preparations.

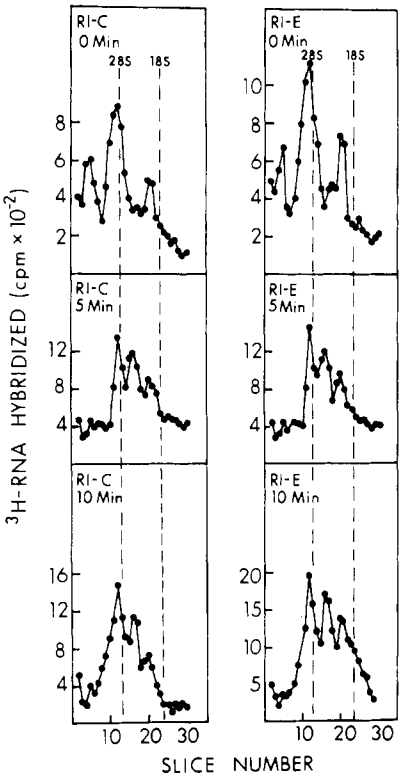

FIGURE 7: Effect of in vitro incubation on *Eco*R1C and E polyadenylated RNAs labeled in vivo. Cultures were labeled with [^{3}H]uridine for 30 min as described in Figure 6. Isolated nuclei were then incubated in the standard RNA synthesis mixture for the indicated times. Polyadenylated RNAs were purified and fractionated by electrophoresis in formamide gels. Equal aliquots of the RNA eluted from each gel slice were then annealed to 1-μg equiv of *Eco*R1 C and E fragments.

RNAs had a size distribution ranging from approximately 13S to 35S.

The in vitro products contained polyadenylated viral RNAs at least as large as the 11S–28S cytoplasmic mRNA population but smaller than the high-molecular-weight pulse-labeled RNAs that have been found in nuclei in vivo (Bachenheimer and Darnell, 1975). The different size distribution of in vitro and in vivo nuclear RNAs might reflect transcriptional differences or alternatively might indicate nuclease activity in isolated nuclei. To consider the latter possibility, cultures were labeled with [^{3}H]uridine in vivo for 30 min beginning 14 h after infection (Brunner and Raskas, 1972). Nuclei were purified, and polyadenylated nuclear RNA was purified from one aliquot. This RNA was fractionated by electrophoresis and annealed to adenovirus 2 DNA (Figure 6, left panel). This RNA preparation contained a high percent of molecules significantly larger than the in vitro products, with a size distribution ranging from 22S to 36S. Additional aliquots of the nuclei labeled in vivo were incubated in vitro in conditions identical to those used for synthesis, except that radioactive UTP was omitted. Polyadenylated RNAs were purified from nuclei that had been incubated for 5 and 10 min (Figure 6, middle panels). Fractionation of the RNA by size and hybridization to viral DNA revealed that incubation resulted in an altered size distribution which gradually approached that of the in vitro products.

Analysis of Nuclear RNAs Transcribed from Genome Map Positions 83–100. The analysis of nuclear RNAs was extended to a defined region of the genome, map positions 83–100. This region of the genome specifies the mRNA for fiber, a major virion polypeptide (Lewis et al., 1975; Mautner et al., 1975). The mRNA is 22S in size (Tal et al., 1974); R-loop mapping has shown that the sequences for this mRNA are specified by map positions 86–92 (Chow et al., 1977a). Nuclear RNAs from this genome region were analyzed using *Eco*R1 fragments E and C which include the sequences in map positions 83–89 and 89–100, respectively.

As in the experiments described in the previous section, cultures were labeled in vivo for 30 min and nuclei were then incubated in vitro in the absence of radioactive nucleotides. At appropriate times, reactions were terminated and polyadenylated RNA was purified. The RNA was subjected to electrophoresis and analyzed by hybridization to DNA fragments. Identical results were obtained when either *Eco*R1 C or E was used as the hybridization probe (Figure 7). RNA from nuclei that had not been incubated in vitro contained major 35S, 28S, and 22S RNA size classes. In vitro incubation resulted in the disappearance of the 35S RNA and the appearance of a new 25S size class.

RNAs labeled in vitro were also analyzed for the size distribution of *Eco*R1 C and E transcripts (Figure 8). Preparations labeled by incubating with [^{3}H]UTP for 10 min were fractionated by electrophoresis and then hybridized. The size classes of *Eco*R1 C and E transcripts were identical, containing major 28S, 25S, and 22S RNAs. These profiles are essentially identical to those obtained when in vivo labeled RNA has been incubated in vitro for 10 min.

Discussion

Previous studies of adenovirus 2 RNA synthesis in isolated nuclei have demonstrated that an RNA polymerase II activity is responsible for transcription of molecules believed to be precursors of viral mRNA (Weinmann et al., 1976). The current studies further analyzed viral RNA synthesis in nuclei isolated late in infection, at 14 h. In vitro transcripts had four properties similar to RNAs synthesized in vivo: (1) a large fraction of the in vitro RNAs were virus specified; (2) nearly all the viral RNAs were transcribed from the *r* strand; (3) the

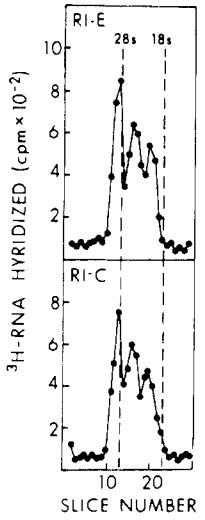

FIGURE 8: Hybridization of polyadenylated nuclear RNA labeled in vitro to EcoR1 fragments C and E. RNA labeled during a 10-min incubation with [^{3}H]UTP was purified, denatured, and subjected to electrophoresis as in Figure 6. Equal samples from the RNAs eluted from each gel slice were hybridized to *Eco*R1 C (lower panel) and E (top panel).

viral transcripts included sequences restricted to the nucleus in vivo as well as sequences that are found in mRNA in vivo; (4) the in vitro RNAs appear to be polyadenylated. RNA labeled with [^{3}H]UTP during synthesis routinely binds to oligo(dT)-cellulose approximately 30%. Since these molecules are labeled with radioactive uridine, the molecules binding to oligo(dT)-cellulose must represent molecules that have undergone template-directed elongation followed by polyadenylation. The above results are similar to those obtained by Vennstrom and Philipson (1977).

Several different studies have provided evidence for a major late promotor located approximately at map position 20 on the *r* strand. It has been suggested that this promotor controls transcription of the major late gene block which extends from position 20 to the right end of the genome (position 100). The relevant studies include analyses of the following: (1) the sequences represented in the large nuclear RNAs pulse labeled in vivo and in vitro (Bachenheimer and Darnell, 1975; Weber et al., 1977); (2) the DNA sequences specifying small nascent RNAs in vivo (Evans et al., 1977); and (3) the effect of UV irradiation on nuclear viral RNA synthesis in vivo (Goldberg et al., 1977, 1978). A one promotor–one terminator model for genome segment 20–100 at late times results in certain predictions for the distribution of primary transcripts in in vitro and in vivo samples. Assuming that incorporation in the isolated nuclei is limited to chain elongation and termination occurs with fidelity, analysis of the in vitro transcripts can provide evidence relevant to such models. The kinetics in our in vitro system do suggest that the nucleotide incorporation represents predominantly elongation of molecules that are in formation at the time of isolation. Other studies have demonstrated directly the absence of detectable initiation of synthesis by RNA polymerase II in nuclear extracts (Gilboa and Aviv, 1976; Vennstrom et al., 1978). The size analysis of transcripts from the right end of the genome suggests that termination is similar in vivo and in vitro (see below).

If there were only one terminator and one promotor for the major rightward gene block, the amount of RNA labeled during in vitro elongation and termination should increase as the right end of the genome is approached. As shown in Figure 5, this result was not obtained. It has been estimated that transcription of the entire adenovirus genome in vivo requires 6 min (Darnell et al., 1967; Greenberg and Penman, 1966). RNAs labeled in vivo for 4 min exhibited a similar distribution to that of the in vitro products. If all segments of the genome positions 20–100 are being uniformly transcribed in vivo and RNA polymerases are uniformly distributed on the template, a 4-min label should yield a uniform distribution across the genome unless a significant degradation occurs. These results suggest that more than one promotor–terminator regulatory element may function for this region of the genome. In fact, several recent studies have presented data suggesting the presence of a second promotor within this region (20–100) (Weinmann and Aiello, 1978; Girvitz and Rainbow, 1978).

A previous study from our laboratory suggested that adenovirus mRNA processing can be studied in isolated nuclei (Bruner and Raskas, 1972). Investigations with other eukaryotic systems have presented evidence for processing of ribosomal RNA, tRNA, and 5S RNA in isolated nuclei as well as with purified RNase III (Marzluff et al., 1973, 1974; Busiello and DiGirolamo, 1975; Gotoh et al., 1974; Ohtsuki et al., 1977). Our analysis of the size distribution of polyadenylated viral RNAs after in vitro incubation has demonstrated specific size changes in viral RNAs. In vitro incubation of nuclei labeled in vivo converted the in vivo transcripts to a different size distribution (Figure 6). The specificity of this conversion was more obvious when transcripts from a defined region of the genome were examined, the DNA segment encoding the fiber polypeptide. The fiber mRNA is 22 S in size and is encoded by portions of *Eco*R1 C and E, two contiguous fragments (Tal et al., 1974; McGrogan and Raskas, 1977). The in vivo nuclear RNAs transcribed from these fragments contained one large 36S size class in addition to 28S and 22S RNAs. This large size class disappeared during incubation in vitro and a new 25S size class appeared. The RNAs larger than the 22S molecules are possible precursors of the functional mRNA. RNAs labeled in vitro contained the three size classes 28S, 25S, and 22S, suggesting that (1) termination of in vitro synthesis reproduces in vivo events and (2) RNA cleavage that occurs in isolated nuclei operates similarly on RNAs labeled in vivo and in vitro. These results suggest that this in vitro nuclear system may be valuable for analyzing coupled transcription and processing of mRNA.

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