Poly(adenylic acid) Sequences in Adenovirus Ribonucleic Acid Released from Isolated Nuclei†

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ABSTRACT: Isolated nuclei prepared from cultures late after adenovirus 2 infection release viral RNA when incubated with ATP, creatine phosphate, and creatine kinase. The released RNA includes molecules the size of mRNA. By the criteria of RNase resistance or binding to Millipore filters, this RNA contains the same amount of poly(A) as found in the polysomal mRNA synthesized late after infection. The RNase-resistant segments are probably identical with the poly(A) of polysomal mRNA, for they bind to Millipore filters in high salt but not low salt buffer, migrate as 6–7 S in polyacrylamide gel electrophoresis, and contain little or no uridine residues. During incubation of isolated nuclei with creatine phosphate and kinase and no exogenous ATP, [^{3}H]ATP was incorporated; little or no [^{3}H]CTP incorporation was detected. The [^{3}H]ATP incorporated into RNA in the nucleus was present in both low molecular weight (4 S) and larger (10–29 S) species. In contrast, the released RNA fraction contained [^{3}H]ATP only in small pieces of RNA, less than 4 S. This result excludes the possibility that additional terminal addition of adenosine to mRNA is required for the release of mRNA molecules *in vitro*.

Recent studies have identified poly(A) segments as a structural feature common to most eukaryotic mRNAs (Lee *et al.*, 1971; Edmonds *et al.*, 1971; Darnell *et al.*, 1971). Poly(A) tracts also occur in mRNAs specified by vaccinia virus (Kates, 1970), a cytoplasmic DNA virus, and by adenovirus 2 (Philipson *et al.*, 1971), a nuclear DNA virus. Late after adenovirus 2 infection (18 hr), when viral macromolecules are the predominant species synthesized (Pina and Green, 1969; White *et al.*, 1969) 60% or more of the mRNA entering polyribosomes is virus specified (Raskas and Okubo, 1971). About 75% of this adenovirus specific RNA contains tracts of poly(A) (Philipson *et al.*, 1971). Recently we have described an *in vitro* system for the study of adenovirus RNA transport (Raskas, 1971) which utilizes nuclei prepared from cultures late after adenovirus 2 infection (18 hr). If cultures are labeled with [^{3}H]uridine for 50 min prior to harvesting, the isolated nuclei release radioactive RNA when incubated in the presence of ATP and an ATP generating system. A substantial portion of the RNA released can be identified as virus specific by hybridization to viral DNA. This viral RNA may be intact mRNA, for it is approximately the size reported for the adenovirus 2 mRNA species (Parsons *et al.*, 1971).

The present report describes the results of investigations into the role of poly(A) sequences in the *in vitro* release reaction. The results presented here demonstrate that released viral mRNA contains essentially the same complement of poly(A) sequences as the polysomal mRNA synthesized *in vivo* 18 hr after infection. Since the poly(A) sequences in hemoglobin mRNA (Burr and Lingrel, 1971), cellular mRNA (Mendecki *et al.*, 1972), and vaccinia virus (Kates, 1970) and poliovirus RNAs (Yogo and Wimmer, 1972) are found at the 3′ terminus of the molecule, we also examined the possibility that release of viral RNA from isolated nuclei is dependent upon the terminal addition of adenosine residues to newly completed mRNA molecules.

Methods

Preparation of Nuclei for in Vitro Reactions. Nuclei were prepared by homogenization of cultured human (KB) cells infected with adenovirus 2 (25–50 plaque-forming units/cell) (Raskas, 1971; Raskas *et al.*, 1970). To obtain nuclei containing radioactive adenovirus RNA, cultures were harvested following labeling with either [^{3}H]uridine (2–6 μCi/ml; 20 Ci/mmol) or [^{3}H]adenosine (2–8 μCi/ml; 20 Ci/mmol) for 50 min beginning 18 hr after infection. Nuclei used to study incorporation of nucleotides were harvested at 18 hr after infection and treated with 0.5% Triton X-100 after homogenization; this procedure reduced the background during incorporations.

In Vitro Reactions with Isolated Nuclei. In vitro release of RNA from nuclei was carried out as described previously (Raskas, 1971). Nuclei were resuspended in KMN[1] buffer containing 10 mM β-mercaptoethanol and incubated at 37° with the addition of 1 mM ATP, 5 mM creatine phosphate, and 30 μg/ml of creatine phosphokinase. At appropriate times, 100-μl aliquots were sampled into 2 ml of KMN buffer containing unlabeled KB nuclei and centrifuged to yield released RNA. Released RNA was collected as CCl_3COOH-precipitable material on Whatman glass fiber (GF/A) filters.

To study the incorporation of nucleotides into macromolecules nonradioactive nuclei were incubated in the same conditions as for RNA release except that the 1 mM ATP was omitted and [^{3}H]ATP (12 μCi/ml; 9.5 Ci/mmol) or [^{3}H]CTP (6 μCi/ml; 22.5 Ci/mmol) was added. Incorporation was assayed by sampling 100-μl aliquots onto 2.4-cm DEAE-cellulose circles (Whatman) which were immediately placed in 5% Na_2HPO_4 containing 0.1 mM ATP. The filters were

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[1] Abbreviations used are: KMN buffer, 8 mM KCl–4 mM NH_4Cl–3 mM $MgCl_2$–10 mM Tris-HCl, pH 7.6; SSC, 0.15 M NaCl–0.015 M sodium citrate; TKM buffer, 50 mM Tris-HCl (pH 7.6)–25 mM KCl–5 mM $MgCl_2$.

washed five times more in 5% Na_2HPO_4, twice in H_2O, twice in 95% EtOH, and then twice in ether. Each wash was for 4 min. With this procedure oligonucleotides containing eight or more residues are retained on the disks (Blatti *et al.*, 1970). The samples were dried and counted in a scintillation counter.

RNA Purification. Following incubation of nuclei that had been labeled *in vivo*, released RNA was obtained as the supernatant after centrifugation for 1800*g*-min in the International PR-2 centrifuge. Carrier cytoplasmic KB RNA (25 μg/ml), 0.5% sodium dodecyl sulfate, and 50 mM EDTA were added prior to extraction at 65° with phenol saturated with 50 mM Tris (pH 7.4). After extraction the RNA was precipitated overnight at 4° with 2 M LiCl (Baltimore, 1966). The RNA precipitated by LiCl was redissolved in 0.1 × SSC and reprecipitated with 2.5 volumes of 95% EtOH and 0.03 volumes of 4 M NaCl.

Following incorporation of [^{3}H]ATP or [^{3}H]CTP by isolated nuclei, the reaction mixture was separated into nuclear and supernatant (or released) fractions by centrifugation for 30,000*g*-min in the Sorvall RC2-B centrifuge. Extraction of nuclear RNA was performed essentially as described by Penman (1966). The nuclei (0.8 ml) from approximately 1.5 l. of infected cells were resuspended in 2–3 ml of high salt buffer (0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris pH 7.4) and passed through a 20-gauge needle ten times to reduce the viscosity of the nuclear gel. Treatment with electrophoretically purified DNase (Worthington) (50 μg/ml) for 30 min at room temperature was followed by dilution with five volumes of 50 mM Tris (pH 7.4)–20 mM EDTA–0.5% sodium dodecyl sulfate. The RNA in the supernatant fraction was extracted after addition of 0.5% sodium dodecyl sulfate, 50 mM EDTA, and 25 μg/ml of carrier RNA. Extraction of both preparations was performed at 65° with phenol saturated with 50 mM Tris (pH 7.4); the initial phenol extraction was followed by reextraction of the interphase and phenol phase with an equal volume of 0.5% sodium dodecyl sulfate–50 mM EDTA–50 mM Tris (pH 7.4). The two aqueous phases were combined, reextracted with an equal volume of phenol, and precipitated with 2.5 volumes of 95% EtOH and 0.03 volumes of 4 M NaCl.

Assays for Poly(A). The resistant fraction of various RNA preparations was obtained by treatment with pancreatic (40 μg/ml) and T_1 (50 units/ml; Calbiochem) ribonucleases in 0.2 M KCl containing 100 μg/ml of poly(A) (Miles Laboratories) (Kates, 1970). Binding of molecules containing poly(A) to nitrocellulose membrane filters (Millipore filters) was performed as described by Lee *et al.* (1971).

As a control, RNA synthesized late after adenovirus 2 infection was prepared from polysomes of cultures pretreated with actinomycin D (0.02 μg/ml) for 30 min and then labeled with [^{3}H]uridine or [^{3}H]adenosine 17–20.5 hr after infection (Raskas and Okubo, 1971). Polysomes were prepared from S30 extracts by centrifugation through a 1 M sucrose layer containing TKM buffer (Bhaduri *et al.*, 1972). Polysomal RNA was resuspended in TKM buffer containing 5% sucrose and extracted at pH 9.0 to preserve poly(A) sequences as described by Lee *et al.* (1971).

Polyacrylamide Gel Electrophoresis. Extracted RNA was analyzed on 2.8% polyacrylamide gel (Raskas and Okubo, 1971). Cytoplasmic [^{14}C]RNA used to provide marker 28S and 18S rRNA was prepared from uninfected KB cells labeled with [^{14}C]uridine (0.25 μCi/ml; 57 Ci/mol) for 24 hr. Electrophoresis was carried out at 5 mA/tube for 3 hr. Gels were then frozen, sliced, solubilized with alkali overnight, and counted in a scintillation counter.

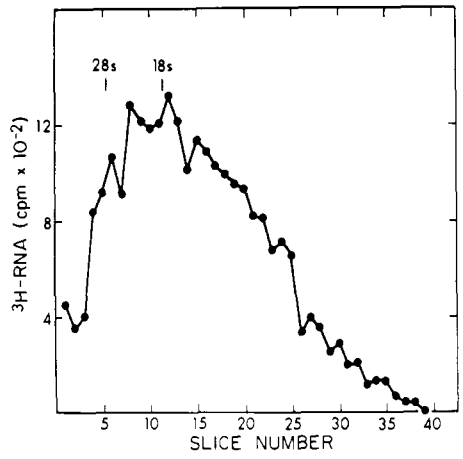

FIGURE 1: Polyacrylamide gel electrophoresis of RNA released from nuclei. Nuclei prepared from infected cultures labeled with [^{3}H]-adenosine *in vivo* were incubated with ATP, creatine phosphate, and creatine kinase as described in the Methods section. Released RNA was extracted, precipitated with LiCl, and analyzed on 2.8% polyacrylamide gels. A parallel gel containing radioactive rRNA provided the reference markers.

Results

The RNA released from isolated nuclei is found as two size classes, low molecular weight 4–6S RNA and higher molecular weight RNA (10–29 S) that corresponds to the sizes anticipated for mRNAs (Raskas, 1971). The 10–29S RNA anneals at least 60% to adenovirus DNA. Since the hybridization efficiency, even in conditions of considerable DNA excess, is rarely more than 75% efficient in a one-step hybridization (unpublished results), nearly all molecules the size of mRNA must be virus specified.

Poly(adenylic acid) Content of mRNA Released from Isolated Nuclei. To further compare the released mRNA to the adenovirus mRNA in polyribosomes, we determined the poly(A) content of the mRNA molecules released *in vitro*. In these experiments infected cultures were labeled with [^{3}H]-adenosine for 50 min prior to harvesting. Isolated nuclei were incubated with ATP and the ATP generating system, and the released RNA was extracted and then precipitated with 2 M LiCl. This treatment selectively precipitates high molecular weight RNA but not low molecular weight RNA nor double-stranded molecules (Baltimore, 1966). Such preparations of released RNA (Figure 1) contained molecules migrating in the size range 10–29 S; low molecular weight RNA was absent as were the high molecular weight adenovirus RNAs which are the precursor species found in the nuclei (Parsons *et al.*, 1971; McGuire *et al.*, 1972).

Preparations of released mRNA were analyzed for poly(A) sequences by two methods. Both the released mRNA and polysomal mRNA synthesized late after adenovirus 2 infection were 12% resistant to pancreatic and T_1 ribonucleases (Table I). Released mRNA labeled with [^{3}H]uridine was less than 0.5% resistant to the same RNase treatment. The second method utilized the ability of Millipore filters to bind selectively molecules that contain poly(A) segments (Lee *et al.*, 1971). Approximately 30% of the [^{3}H]adenosine residues in released mRNA were in molecules that bound to Millipore filters (Table II); nearly 40% of polysomal mRNA synthesized late after infection was bound. Control rRNA did not bind to the membranes under the same conditions. The RNase resistant fraction of released mRNA bound to

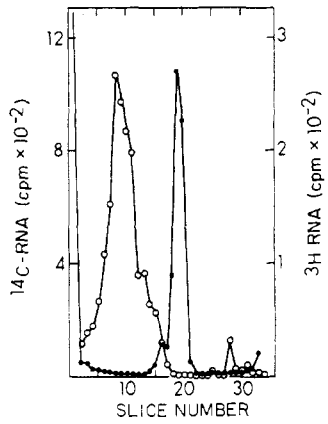

FIGURE 2: Polyacrylamide gel electrophoresis of RNase-resistant fraction of released RNA. RNA ([^{3}H]adenosine) released from isolated nuclei was treated with RNase, precipitated, and bound to Millipore filters as described in Table III. Bound RNA was eluted by incubation in 1 mM Tris (pH 7.6)–0.25% sodium dodecyl sulfate for 1.5 hr at 0°. The eluted RNA was then analyzed by electrophoresis for 3 hr at 5 mA/gel on 7.2% polyacrylamide gels. On a parallel gel cytoplasmic RNA ([^{14}C]uridine) was analyzed to provide a 4S reference marker. (○) [^{3}H]RNA, (●) [^{14}C]RNA.

Millipore filters 76% in high salt but only 8% in low salt buffer (Table III). The resistant material migrated as 6–7 S, slower than 4S tRNA during polyacrylamide gel electrophoresis (Figure 2).

Incorporation of [^{3}H]ATP by Isolated Nuclei. Since RNA release from isolated nuclei is stimulated by ATP and the released mRNA contains poly(adenylic acid) sequences, we investigated the possibility that the ATP is required for synthesis of poly(adenylic acid) sequences. Unlabeled nuclei were isolated from infected cells 18 hr after infection and incubated *in vitro* with radioactive triphosphates. Incorporation of [^{3}H]-ATP was not detected in trial experiments which utilized the same reaction conditions used for release of RNA from nuclei. Since the specific activity of the ATP may have been too low to detect synthesis of a relatively small number of nucleotides, we examined the requirements for the release reaction to determine if these experiments could be performed without adding 1 mM exogenous ATP. When the kinase and phosphate were omitted but 1 mM ATP was present, the release reaction was inhibited 70% (Figure 3). In the absence of ATP, but with creatine phosphate and kinase, RNA release occurred to a significant extent, as much as 60% as the control sample. In subsequent experiments nuclei were incubated in the presence of creatine phosphate, creatine kinase and only the radioactive triphosphate; then [^{3}H]ATP incorporation was detected (Figure 4) whereas there was very little or no incorporation of [^{3}H]CTP. The incorporation of [^{3}H]ATP was complete after 10–15-min incubation and was dependent on the presence of the regenerating system, possibly because nuclei contain ATPase activity (Siebert and Humphrey, 1965).

Analysis of Macromolecules Labeled during [^{3}H]ATP Incorporation. To characterize the macromolecules synthesized during incubation of isolated nuclei, the reaction mixture was incubated with [^{3}H]ATP for 15 min and then separated into two fractions, nuclear and supernatant (or released) material; 70% or more of the incorporated [^{3}H]ATP was found in the supernatant. The RNA in each fraction was extracted and analyzed by polyacrylamide gel electrophoresis (Figure 5). The supernatant material was very small in size, less than 4 S. In contrast the nuclei contained a low molecular weight com-

TABLE I: RNase Resistance of RNA Released from Isolated Nuclei.[a]

RNA Sample	Input cpm	Resistant cpm	% Resistant
Released [^{3}H]adenosine	8072	983	12
Polysomal [^{3}H]adenosine	3440	425	12
Released [^{3}H]uridine	3950	20	0.5

[a] Released RNA was prepared from nuclei that had been labeled with [^{3}H]adenosine or [^{3}H]uridine prior to harvesting. Following incubation of nuclei for 20 min with ATP, creatine phosphate, and creatine kinase, released RNA was extracted from the supernatant fraction and precipitated by LiCl as described in the Methods section. Polysomal RNA was prepared from the polyribosomes of cultures that had been labeled with [^{3}H]adenosine in the presence of 0.02 μg ml of actinomycin from 17 to 20.5 hr after adenovirus 2 infection. Each preparation was treated with pancreatic and T_1 RNase as described in the text. Radioactivity was determined by precipitation with CCl_3COOH.

TABLE II: Binding of Released RNA to Millipore Filters.[a]

Sample	Input cpm	Bound cpm	% Bound
Released RNA	4557	1442	31
Released RNA	8024	2294	28
Polysomal RNA	5192	2099	40
rRNA	5236	39	0.7

[a] Released RNA ([^{3}H]adenosine) was obtained from isolated nuclei by incubation *in vitro* followed by extraction and precipitation with LiCl. Control polysomal RNA ([^{3}H]-adenosine) was prepared from infected cultures and rRNA ([^{3}H]uridine) from uninfected cultures. The released RNA and polysomal RNA were then tested for ability to bind to Millipore filters as described by Lee *et al.* (1971). For details, see Methods section and Table I.

TABLE III: Binding of RNase-Resistant RNA to Millipore Filters.

Binding Conditions	Input cpm	Bound cpm	% Bound
500 mM KCl–1 mM $MgCl_2$–10 mM Tris (pH 7.6)	1280	971	76
50 mM KCl–1 mM $MgCl_2$–10 mM Tris (pH 7.6)	1280	104	8

[a] Released RNA ([^{3}H]adenoisne) was treated with pancreatic and T_1 RNase as described in the Methods section. RNase-resistant RNA was collected by alcohol precipitation, resuspended in H_2O, and then diluted into buffers containing either high or low salt concentrations. Binding to Millipore filters was performed according to Lee *et al.* (1971).

TABLE IV: Alkali Sensitivity of [^{3}H]ATP Incorporated by Nuclei.[a]

	CCl_3COOH-Precipitable cpm
Untreated	352
80° 2 hr	13
37° overnight	16

[a] Isolated nuclei from infected cultures were incubated for 20 min in the presence of [^{3}H]ATP, creatine phosphate, and creatine kinase as described in the Methods section. Following the incubation nuclear RNA was purified and aliquots tested for resistance to treatment with 0.3 N NaOH as indicated above. After exposure to alkali the samples were neutralized, carrier calf thymus DNA was added (50 μg/ml), and CCl_3-COOH-precipitable radioactivity was determined.

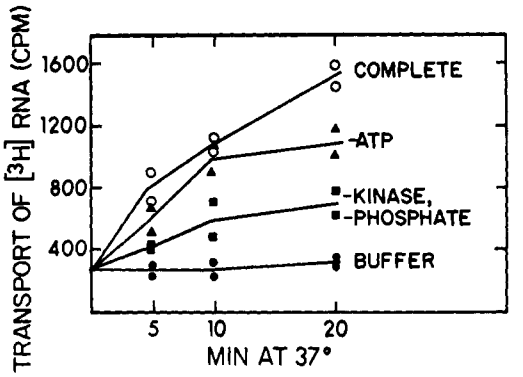

FIGURE 3: Dependence of *in vitro* release reaction on ATP, creatine phosphate, and creatine kinase. Nuclei prepared from infected cultures labeled with [^{3}H]uridine for 50 min were incubated *in vitro* and sampled at the indicated times to measure release of RNA from nuclei. Reactions were carried out in KMN buffer alone (●), with all three components, ATP, creatine phosphate, and creatine kinase (○), with addition of ATP alone (■), or with creatine kinase and phosphate and no ATP (▲).

ponent but also included RNA large enough to be mRNA. The relative amounts of these two size classes of nuclear RNA varied; in some experiments as much as 50% of the [^{3}H]ATP was found in molecules of the size range 10–28 S.

The macromolecules obtained by phenol extraction of the nuclear fraction were characterized further. The product labeled with [^{3}H]ATP was alkali labile (Table IV). A large somal mRNA synthesized late after adenovirus 2 infection were 9% resistant. In one instance some incorporation of [^{3}H]CTP was detected during incubation of the isolated nuclei. This RNA was less than 4% resistant under identical conditions of incubation (Table V).

Discussion

The use of isolated nuclei to study mRNA processing and transport offers many analytical advantages if the relationship of *in vitro* RNA release to *in vivo* mRNA transport can be ascertained. Although there is not yet any direct evidence for the relevance of *in vitro* release to *in vivo* transport, the data presented here are consistent with the conclusion that mRNA released *in vitro* is essentially intact. Several methods of analysis showed that released mRNA contains the same amount of poly(adenylic acid) sequences as the polysomal mRNA synthesized late after adenovirus 2 infection: Released RNA labeled with [^{3}H]adenosine was 10–12% resistant to pancreatic and T_1 ribonucleases, a value similar to that obtained for polysomal mRNA in our experiments and in agreement with that reported by Philipson *et al.* (1971) for adenovirus mRNA synthesized late in the infection. The resistant fraction appears identical to the poly(A) segment of adenovirus mRNA in polyribosomes, for it does not contain significant quantities of uridine, it binds to Millipore filters in high salt buffer, and migrates as 6–7 S during electrophoresis. Although the released RNA is derived from nuclei *in vitro*, these properties of the RNase resistant material differentiate it from the double-stranded RNA recently found in heterogeneous nuclear RNA (Jellinek and Darnell, 1972).

The extent of binding to millipore filters is a particularly useful indication of the intactness of RNA, for a single nick will prevent the attachment of a large segment of the molecule. We found that 30% of the released mRNA bound to Millipore filters as compared to 40% for mRNA molecules isolated from polyribosomes after infection. Philipson *et al.*

fraction of the product, 25–40%, was resistant to pancreatic and T_1 ribonucleases (Table V); control samples of poly-

TABLE V: RNase Resistance of Nucleotides Incorporated by Nuclei.[a]

Expt	RNA Source	Input cpm	% Resistant
A	*In vitro* nuclear [^{3}H]adenosine	759	39.6
	In vivo polysomal [^{3}H]adenosine	904	9.0
B	*In vitro* nuclear [^{3}H]adenosine	1010	24.6
	In vitro nuclear [^{3}H]cytosine	890	3.9

[a] Nuclear RNA was purified from isolated nuclei incubated for 20 min with creatine phosphate, creatine kinase, and [^{3}H]ATP or [^{3}H]CTP. Control *in vivo* polysomal RNA was prepared from infected cultures labeled with [^{3}H]adenosine in the presence of 0.02 μg/ml of actinomycin. RNA samples were treated with pancreatic and T_1 RNase (see Methods) and CCl_3COOH-precipitable radioactivity was determined.

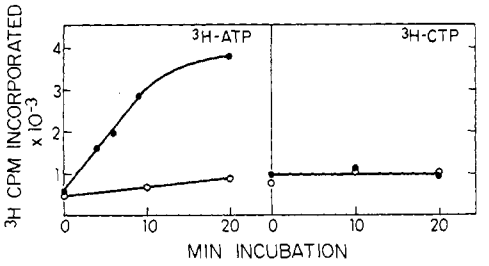

FIGURE 4: Incorporation of ribose triphosphates by isolated nuclei. Nonradioactive nuclei were prepared from cultures 18 hr after infection. Nuclei were incubated in KMN buffer with [^{3}H]ATP or [^{3}H]CTP and assayed for incorporation of nucleotides by sampling 100-μl aliquots onto DEAE-cellulose circules at the indicated times. In each instance one reaction tube contained creatine phosphate and kinase (●) and the other did not (○).

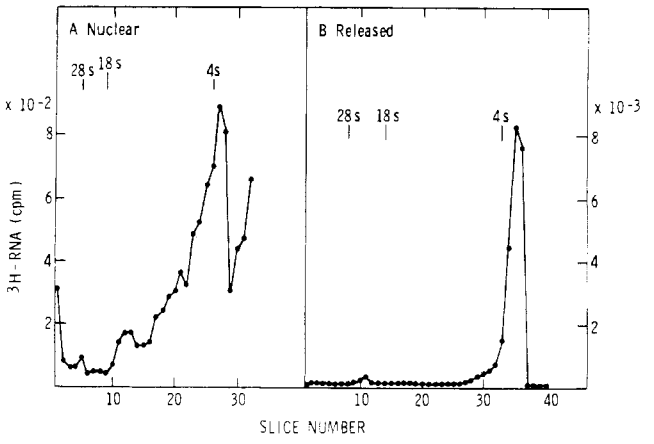

FIGURE 5: Polyacrylamide gel electrophoresis of RNA synthesized by isolated nuclei. Isolated nuclei from infected cultures were incubated with [^{3}H]ATP (20 μCi/ml) for 20 min and then separated into nuclear and released (supernatant) fractions as described in the Methods section. Each preparation was purified by phenol extraction, precipitated with EtOH, and analyzed on 2.8% polyacrylamide gels; a parallel gel with radioactive rRNA provided the 28S, 18S, and 4S reference positions.

(1971) had reported that 60–80% of adenovirus RNA in polyribosomes contained poly (A) tracts. Since [^{3}H]poly(A) was bound only 30% in our experiments, the variation in extent of binding probably was caused by variations in the binding efficiency of Millipore filters.

In other experiments we have shown (Brunner and Raskas, 1972) that prior to release from nuclei viral mRNA is cleaved from the high molecular weight precursor form to discrete species the size of the polysomal mRNAs (Parsons *et al.*, 1971). Further evidence for the integrity of released mRNA has been obtained by Ishikawa *et al.* (1972) who found that RNA released from rat liver nuclei by incubation with ATP can be incorporated into polyribosomes *in vitro*.

We found that during incubation of nuclei incorporation of [^{3}H]ATP into RNA could be detected if the nonradioactive 1 mM ATP were omitted from the reaction. Similar observations were made by Kato and Kurokawa (1970) with nuclei from various mammalian tissues. Examination of RNA release from radioactive nuclei showed that under these conditions less RNA is released (Figure 3), but the same size distribution of released RNA is obtained (data not shown). Presumably the reaction proceeds because the nuclei contain endogenous adenosine nucleotides; we determined the intranuclear adenosine concentration by sequential treatment of nuclear extracts with alkaline phosphatase and adenosine deaminase and found the adenosine concentration to be 0.1 mM at the concentration of nuclei (10^7/ml) used in these experiments. From these data and the specific activity of the [^{3}H]ATP, the *in vitro* incorporation is calculated to be nearly 10^5 adenosine nucleotides/nucleus. Since 20–30% of the incorporated [^{3}H]-ATP was found in the nuclei after incubation and 30% of this RNA was resistant to RNase (Table V), each nucleus contained approximately 10,000 adenosine residues incorporated into poly(A). Vaccinia virus cores prepared by detergent treatment of virions also synthesize poly(A) in the absence of other nucleotide substrates (Kates and Beeson, 1970); with this system 30,000 nucleotides are incorporated into poly(A) per core particle.

The poly(A) segments in hemoglobin mRNA (Burr and Lingrel, 1971), cellular RNA (Mendecki *et al.*, 1972), and vaccinia virus (Kates, 1970) and poliovirus RNAs (Yogo and Wimmer, 1972) are found at the 3′ terminus of the molecule. Although released mRNA contains poly(A) segments in the frequency found for mRNA *in vivo*, our data show that terminal addition of adenosine residues is not required for RNA release. If addition of adenosine to the 3′ terminus were required for RNA release, it should be found in the mRNA released from nuclei. However, the incorporated [^{3}H]ATP found in the released RNA was present only as very small fragments (Figure 5). This result excludes the terminal addition of poly(A) as a requirement for RNA release *in vitro* unless the nuclei contain an inaccessible pool of nonradioactive nucleotides which are used for poly(A) synthesis. At present it remains difficult to exclude a need for incorporation of [^{3}H]-ATP into small RNA species or other molecules. It should be noted that we are not yet able to determine if poly(A) sequences are required for RNA transport *in vitro*, for the results of others (Darnell *et al.*, 1971) indicate that nuclei do not contain pieces of poly(A), and our study indicates the mRNA in isolated nuclei already contains poly(A) segments.

Following incubation with [^{3}H]ATP the nuclei do retain some [^{3}H]adenosine in molecules large enough to be mRNA (Figure 5). Since the nuclei contain limited amounts of nucleotides, these adenosine residues may be extensions of growing RNA chains or may indeed represent poly(A) synthesis. These possibilities could be distinguished if chain growth were inhibited by preincubating in the presence of actinomycin D prior to the addition of [^{3}H]ATP. Since we have found much of the [^{3}H]ATP incorporated by nuclei to be poly(A) synthesis, this synthesis, whether in small or large molecules, may reflect the activity of the enzyme responsible for poly(A) addition or alternatively may simply reflect the ability of RNA polymerases to synthesize free or primer-linked poly(A) when ATP is the only triphosphate present (Chamberlin and Berg, 1962; Edmonds and Abrams, 1960, 1962; Venkataraman and Mahler, 1963; Gurgo *et al.*, 1970).

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Isolation of Poly(adenylic acid)-Rich Ribonucleic Acid from Mouse Myeloma and Synthesis of Complementary Deoxyribonucleic Acid†

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ABSTRACT: An RNA fraction containing poly(adenylic acid) sequences has been isolated from membrane-bound polysomes of mouse myeloma tumor. Annealing to a poly(thymidylic acid)-cellulose column, significant RNase-resistance (20%), and the size of the RNase-resistant fragment (7 S) all suggest that this RNA fraction contains large tracts of poly(adenylic acid)-rich sequences. This is confirmed by the finding of 80% AMP in the RNase-resistant fraction. Conditions of temperature and ionic strength for chromatography on poly(thymidylic acid)-cellulose are established for optimal recovery of RNA containing poly(adenylic acid) with minimal contamination by ribosomal RNA. The isolated poly(adenylic acid)-rich fraction functions as an efficient RNA template for the synthesis of complementary DNA with the RNA-dependent DNA polymerase of avian myeloblastosis virus. This reaction is completely dependent upon added RNA as template, and oligothymidylic acid as primer. Priming activity is inversely related to the size of the oligothymidylic acid. Density equilibrium centrifugation of the native product shows that some of the radioactivity is associated with RNA. However, after denaturation, all the radioactivity bands as DNA. The average size of the product synthesized, as determined by alkaline sucrose sedimentation, depends on the concentration of the deoxyribonucleoside triphosphates used in the reaction. The specificity of the product synthesized is demonstrated by hybridization.

One of the intriguing questions concerning the immune response is the generation of antibody diversity. As yet, there are no experimental data to decide between the two main theories proposed: the germ line theory and the theories involving somatic mutation and recombination (for review see Smith *et al.*, 1971). A measure of the number of genes for immunoglobulin chains in cells of different tissues, including the genes for the variable part, could answer that question. Such a measure could be achieved by DNA–DNA hybridization under conditions of large DNA excess, using a "Cot" analysis (Britten and Kohne, 1968; Gelb *et al.*, 1971). One advantage of such an approach is that *in vitro* synthesized DNA could be obtained at high enough specific activities to give meaningful data. In addition, DNA–DNA hybridizations are less susceptible to degradation at high temperature when compared to those performed with RNA.

After the discovery of the RNA-dependent DNA polymerase in tumor viruses (Baltimore, 1970; Temin and Mizutani, 1970), and the subsequent demonstration that it could use 9S hemoglobin (Hb)[1] mRNA as template for complemen-

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[1] Abbreviations used are: Hb, hemoglobin; cDNA, complementary deoxyribonucleic acid; medium A-100, 50 mM Tris-HCl (pH 7.6)–100 mM KCl–5 mM $MgCl_2$; AMV, avian myeloblastosis virus; SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate); DEP, diethylpyrocarbonate; poly(A), poly(adenylic acid).