Association of the Polyadenylate Segment of Messenger RNA with Other Polynucleotide Sequences in Mouse Sarcoma 180 Polyribosomes†

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ABSTRACT: Limited digestion of polysomal RNA with pancreatic ribonuclease releases a structure consisting of poly(A) associated with other polyribonucleotide sequences. This complex can be purified by oligo(dT)-cellulose chromatography. Heating or formamide treatment causes the dissociation of fragments free of poly(A) from the poly(A)-containing components. The two types of fragments tend to reassociate under annealing conditions, and this association is prevented by poly(U). Control experiments indicate that this structure is not an artifact generated during the manipulations. The same structure can be obtained by limited RNase digestion of polyribosomes, followed by deproteinization. The results suggest that the mRNA in polyribosomes may have a defined configuration caused by the interaction of the poly(A) sequence with another segment of the RNA.

Most mammalian mRNA molecules are terminated by a long poly(A)[1] sequence (Lim and Canellakis, 1970; Darnell et al., 1971; Edmonds et al., 1971; Lee et al., 1971). A similar sequence is associated with the heterogeneous nuclear RNA (Darnell et al., 1971; Edmonds et al., 1971; Mendecki et al., 1972). Although the poly(A) segment has provided a precise criterion for the identification and isolation of mRNA, its physiological role is unknown. The discovery of poly(A) in the mRNA of viruses which replicate exclusively in the cytoplasm has suggested a cytoplasmic function (Armstrong et al., 1972; Johnston and Bose, 1972; Yogo and Wimmer, 1972). However, the poly(A) segment does not appear to be essential for the translational process (Bard et al., 1974; Sippel et al., 1974; Soreq et al., 1974; Williamson et al., 1974). It has been suggested that poly(A) could be involved in the determination of mRNA secondary structure (Rosenfeld et al., 1972). A well-defined secondary structure has been described in phage mRNA (Steitz, 1969; Ricard and Salzer, 1974), and indirect studies have also suggested the existence of secondary structure in mammalian mRNA (Lanyon et al., 1972; Palacios et al., 1972). Participation of poly(A) in mRNA secondary structure would imply an association with other sequences in the molecule.

In this communication we report the isolation of a structure from mouse sarcoma 180 ascites cells consisting of poly(A) in association with other polyribonucleotide sequences. This structure is detected by limited ribonuclease digestion of polysomal RNA or polysomes followed by isolation of the poly(A)-containing fragments. The structure may be the product of interaction between the terminal poly(A) sequence and other regions of the mRNA.

Materials and Methods

Cell Incubation, Polysome Preparation, and RNA Extraction. Detailed procedures for the maintenance, labeling, and disruption of mouse sarcoma 180 ascites cells have been described previously (Lee et al., 1971; Mendecki et al., 1972). In various experiments, cells were labeled by incubation for 2 hr with 5 μCi/ml of [2,8-^{3}H]adenosine (32 Ci/mmol), 5 μCi/ml of [5,6-^{3}H]uridine (50 Ci/mmol), 1 μCi/ml of [8-^{14}C]adenine (60 mCi/mmol), or 10 μCi/ml of [^{3}H(G)]hypoxanthine (15 Ci/mmol) (New England Nuclear, Boston, Mass.). Cell suspensions were supplemented with 0.05 μg/ml of actinomycin D in order to prevent the labeling of rRNA (Penman et al., 1968). In some experiments cells suspensions were incubated with [^{3}H]uridine and [^{3}H]hypoxanthine in order to label all the purine and pyrimidine bases in RNA. Procedures for the preparation of sarcoma 180 polysomes by Mg^{2+} precipitation from cell lysates (Mendecki et al., 1972) and extraction of polysomal RNA in the presence of pH 9.0 Tris-HCl (Brawerman et al., 1972) have been previously described.

RNase Hydrolysis of Polysomes and Polysomal RNA. Mild hydrolysis was carried out in incubation mixtures containing 40 A_{260} units/ml of polysomes or polysomal RNA and 1 μg/ml of pancreatic ribonuclease A (RNase) in the presence of 50 m*M* Tris-HCl (pH 7.6)–100 m*M* KCl–5 m*M* $MgCl_2$ for 16 min at 0°. Digested RNA fragments were recovered by phenol extraction in the presence of 0.1 *M* Tris-HCl (pH 9.0) and 0.5% sodium dodecyl sulfate (Brawerman et al., 1972). The aqueous phases were reextracted three times with fresh phenol and precipitated in the cold by addition of 0.05 volume of 2 *M* NaCl and 2.5 volumes of 95% ethanol.

Isolation of the Poly(A)-Containing Structure. RNA fragments containing poly(A) sequences were selected by adsorption of the deproteinized digests on Millipore filters in the presence of 10 m*M* Tris-HCl (pH 7.6)–500 m*M* KCl–1 m*M* $MgCl_2$ (Lee et al., 1971). Adsorbed RNA was eluted from the filters with 100 m*M* Tris-HCl (pH 9.0)–0.1% dodecyl sulfate (Mendecki et al., 1972).

Poly (A)-containing RNA fragments were purified by chromatography on columns of oligo(dT)-cellulose. In this

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[1] Abbreviations used are: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); oligo(dT), oligo(deoxyribothymidylic acid).

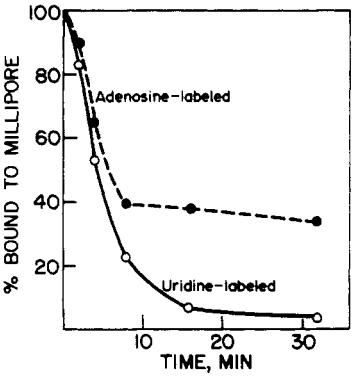

FIGURE 1: Mild RNase hydrolysis of polysomal RNA. Polysomal RNA preparations labeled with [^{3}H]adenosine or [^{3}H]uridine were digested with 1 μg/ml of RNase at 0° in 50 m*M* Tris-HCl (pH 7.6)-100 m*M* KCl-5 m*M* $MgCl_2$. Aliquots were removed at various times during the reaction and filtered through Millipore after dilution in 5 ml of 10 m*M* Tris-HCl (pH 7.6)-500 m*M* KCl-1 m*M* $MgCl_2$. Values are expressed as percent of original Millipore-bound radioactivity. (—) Uridine radioactivity; (- - -) adenosine radioactivity.

procedure the fragments eluted from Millipore were neutralized, diluted in 50 m*M* Tris-HCl (pH 7.6)-200 m*M* NaCl-5 m*M* $MgCl_2$-0.1% dodecyl sulfate, and loaded on 5 × 10 mm columns equilibrated with the same buffer. The columns were operated at room temperature. After washing with 10 ml of application buffer, followed by 5 ml of 10 m*M* Tris-HCl (pH 7.6)-20 m*M* NaCl-1 m*M* $MgCl_2$-0.1% dodecyl sulfate, the poly(A)-containing fragments were eluted from the column with 10 m*M* Tris-HCl (pH 7.6)-0.1% dodecyl sulfate. Column fractions were collected, aliquots were processed for measurements of acid-insoluble radioactivity as described previously (Lee et al., 1971), and the appropriate fractions were pooled and stored at −20°.

Poly(U)-Filter Assays. Poly(U)-glass fiber filters (Whatman GF-C) were prepared as described by Sheldon et al. (1972a). Radioactive RNA samples, diluted in a large excess of 10 m*M* Tris-HCl (pH 7.6)-100 m*M* NaCl (binding buffer), were applied on the filters. These were then washed with 20 ml of binding buffer, dried, and counted as described above.

Polyacrylamide Gel Electrophoresis. RNA fragments were mixed with marker [^{14}C]tRNA (Miles Laboratories, Kankakee, Ill.) and subjected to electrophoresis in 7.5% polyacrylamide gels (5 cm long). Ethylene diacrylate was used as cross-linking agent. The electrophoreses were carried out at room temperature at 1 mA cm^{-1} for 150 min as described previously (Kwan and Brawerman, 1972). The gels were sectioned into 1-mm slices. Two adjacent slices were either counted directly after dissolving in 1 *N* NH_4OH (24-hr incubation at 37°) or counted after elution of the RNA by a 24-hr incubation in 50 m*M* Tris-HCl (pH 7.6)-50 m*M* KCl-1 m*M* $MgCl_2$. In either case the samples were suspended in 5 ml of Triton X-100 scintillation mix and counted as described previously (Lee et al., 1971).

Results

Limited Hydrolysis of Polysomal RNA. Pure poly(A) sequences are released from mRNA molecules after exhaustive digestion of polysomal RNA (Mendecki et al., 1972). The poly(A) can be separated from the other digestion products by virtue of its capacity to bind to Millipore filters (Lee et al., 1971). We have developed conditions for limited hydrolysis of polysomal RNA as a means to study

Table I: Adsorption on Millipore of RNase-Treated Polysomal RNA.[a]

Type of Label in RNA	Percent of Total Radioactivity Bound to Millipore			
	1 μg/ml of RNase		20 μg/ml of RNase	
	0°	30°	0°	30°
Adenine	36	33	30	30
Uridine	5.6	1.0	0.6	0.7

[a] Polysomal RNA was digested with RNase for 16 min and recovered by phenol extraction, and aliquots were adsorbed to Millipore filters as described under Materials and Methods. Values expressed as percent of total radioactivity bound to filters from undigested aliquots of polysomal RNA.

Table II: Interaction of Millipore-Bound RNA Fragments with Poly(U)–Glass Fiber Filters.[a]

Type of Filter	Adenine Radioactivity	Uridine Radioactivity
Millipore	414	537
Poly(U)–glass fiber	402	336

[a] RNA fragments prepared by RNase (1 μg/ml) digestion at 0° and absorbed on Millipore filters as in Table I. Material eluted from the filters was precipitated with ethanol and used for the binding experiments. Values expressed as amounts of radioactive material (counts/min) retained on the filter.

the possible interaction of the poly(A) segment with other polynucleotide sequences. In order to detect the residual polynucleotide sequences associated with the poly(A) in partial digests, RNA labeled with both [^{14}C]adenine and [^{3}H]uridine was used. As shown in Table I, digestion of the RNA at 0° in the presence of relatively low levels of enzyme leaves a significant amount of uridine-labeled material capable of binding to Millipore. This residual material is not particularly resistant to RNase. This is indicated by the fact that more extensive enzyme treatments leave little, if any, uridine-labeled fragments capable of binding to Millipore.

The data in Figure 1 show that the amount of uridine-labeled polynucleotides that can bind to Millipore decreases rapidly during the course of RNA digestion. The small amount of this material left after 16 min of incubation appears to be degraded more slowly.

Interaction of RNA Fragments with Poly(U). It had been assumed in the above experiments that the uridine-labeled RNA fragments were binding to Millipore by virtue of their association with poly(A). Since the basis for the binding of this sequence to Millipore filters is not understood, it was necessary to use a more specific criterion for the presence of poly(A) in the fragments. Thus the digestion products adsorbed to Millipore were eluted and examined for the capacity to interact with poly(U) coupled to glass fiber filters (Sheldon et al., 1972a). As shown in Table II, about 60% of the uridine-labeled components adsorbed on Millipore filters were retained on the poly(U) filters. It is apparent that some of the RNA fragments contain both pyrimidine nucleotides and poly(A). The data also indicate that at least a portion of the poly(A) segment in the fragments is available for complementary base pairing with poly(U). The RNA fragments that do not interact with the poly(U) apparently do not contain poly(A). Their binding to Millipore indicates

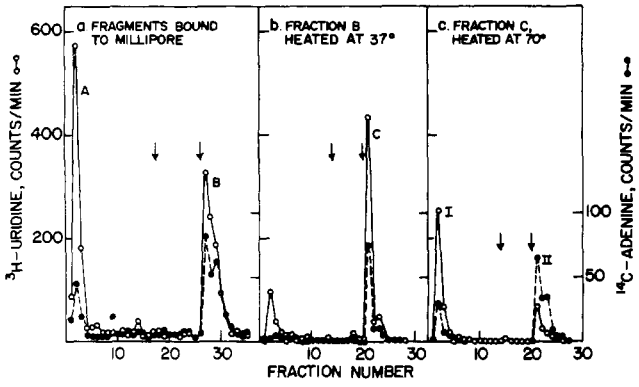

FIGURE 2: Oligo(dT)-cellulose chromatography of polysomal RNA fragments. Polysomal RNA fragments, labeled with [^{3}H]uridine and [^{14}C]adenine and prepared by mild RNase digestion (see Materials and Methods), were enriched in poly(A)-containing components by adsorption on Millipore. (a) Material eluted from the filters was subjected to oligo(dT)-cellulose chromatography; (b) fraction B, which represents the material bound to oligo(dT), was incubated for 5 min at 37° in 10 m*M* Tris (pH 7.6)–10 m*M* KCl–1 m*M* $MgCl_2$–0.1% sodium dodecyl sulfate, cooled quickly, then fractionated again on oligo(dT)-cellulose; (c) material adsorbed in (b) (fraction C) was heated at 70° for 5 min in same buffer as in (b), cooled quickly, then subjected to oligo(dT)-cellulose chromatography. (- - -) [^{14}C]Adenine radioactivity; (—) [^{3}H]uridine radioactivity.

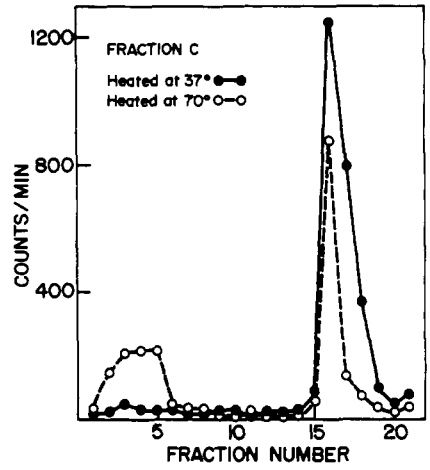

FIGURE 3: Effect of heating on the interaction of poly(A)-containing RNA fragments with oligo(dT)-cellulose. Aliquots of fraction C (see Figure 2) were heated to 37° (—) or 70° (- - -) as outlined in Figure 2 prior to oligo(dT)-cellulose chromatography. Polysomal RNA from which the fragments were derived was labeled with [^{3}H]uridine and [^{3}H]hypoxanthine.

Table III: Binding to Poly(U) Filters of RNA Fragments Eluted from the Oligo(dT)-Cellulose Column.[a]

Type of Label in RNA	Total	Amount Bound to Poly(U) Filter: No Prior Heating	Amount Bound to Poly(U) Filter: Prior Heating at 70°
Adenine	70	70	61
Uridine	225	149	57
U/A	3.21	2.13	0.93

[a] Material purified by oligo(dT)-cellulose chromatography was precipitated with ethanol and 0.1 *M* NaCl at −20°. Precipitates were dissolved in binding buffer prior to poly(U) filtration (see Materials and Methods). Values expressed as radioactivity (counts/min) retained on filters.

that this selection procedure is not strictly specific for poly(A).

Isolation of Poly(A)-Containing Fragments. The poly(A)-containing structures were isolated in larger quantities from the products of limited digestion by oligo(dT)-cellulose chromatography. The elution profile of fragments obtained from double-labeled polysomal RNA is shown in Figure 2a. Adsorption on Millipore was used first as an enrichment step. A substantial portion of the uridine-labeled fragments did not interact with oligo(dT)-cellulose. The rest was firmly bound to the column, and could be eluted only at a low ionic strength required for elution of the poly(A) segments. A second cycle of chromatography after incubation of the eluted poly(A)-containing fragments at 37° led to release of some of the uridine-labeled components from the poly(A)-containing structure (Figure 2b). The remainder, however, is firmly associated with the poly(A) segments, since no further material is released after incubation at 37° and an additional cycle of chromatography (Figure 3).

When the material purified by the two cycles of oligo(dT)-cellulose chromatography was heated at 70°, a large portion of the uridine-labeled material was released from its association with the poly(A) as indicated by its failure to interact with oligo(dT) (Figures 2c and 3). The same results were obtained when the purified material was analyzed by binding to poly(U)–glass fiber filters (Table III). It can be concluded that the structures isolated by oligo(dT)-cellulose chromatography contain polynucleotide fragments devoid of poly(A) associated by noncovalent interactions with poly(A)-containing fragments. Some of the uridine-labeled material remains associated with the poly(A) even after the heat treatment (Figure 2c, Table III). The nature of this association is examined in subsequent sections.

Detection of the Poly(A)-Containing Structure in Polysomes. The isolation of the structure described above indicated that the poly(A) segment in our polysomal mRNA preparations might be associated by noncovalent linkages with another polynucleotide sequence, possibly in the same RNA molecule. Such an association, however, could have been generated during the phenol treatment used to deproteinize the polysomes. Aggregation of RNA components during extraction with phenol has been reported by various investigators (Monier et al., 1962; Asano, 1965; Staehelin et al., 1964; Brawerman et al., 1965; Hayes et al., 1966; Weissman et al., 1968; MacNaughton et al., 1974). Annealing between complementary sequences would be particularly likely to occur if the sequences were on the same RNA molecule. In order to examine this possibility, the RNase treatment was applied to polysomes before deproteinization. Any segments with complementary sequences not already in duplex structures would have become separated during this treatment, and annealing during the subsequent phenol treatment would have been less likely to occur. As can be seen in Figure 4, this procedure yielded the same kind of poly(A)-containing structure as that derived from digests of polysomal RNA. While this experiment provided support for the occurrence of the structure in polysomes, more conclusive evidence would require knowledge of the rates of intermolecular annealing under the conditions of the phenol treatment.

Another control experiment was designed to ensure that the RNA fragments not associated with poly(A) are effectively removed by our purification procedure. Polysomal RNA labeled with [^{3}H]adenosine, from which the poly(A)-

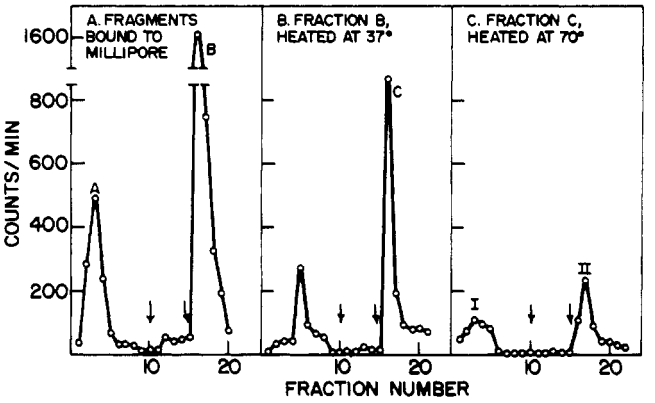

FIGURE 4: Oligo(dT)-cellulose chromatography of RNA fragments derived from polysomes. Polysomes were digested with RNase under mild conditions as described under Materials and Methods. For other details see Figure 2. Polysomes were originally labeled with [^{3}H]uridine and [^{3}H]hypoxanthine.

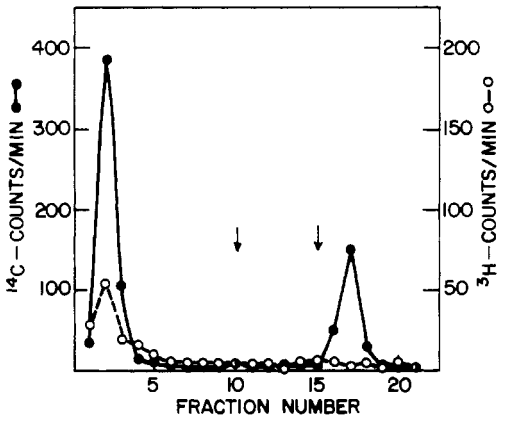

FIGURE 5: Oligo(dT)-cellulose chromatography of mixed RNA fragments derived from polysomes and polysomal RNA. Polysomal RNA labeled with [^{3}H]adenosine, from which poly(A)-containing species were removed by oligo(dT)-cellulose chromatography, was mixed with [^{14}C]adenine-labeled polysomes. The mixture was digested with RNase under mild conditions as in Figure 4, phenol-extracted, Millipore-bound and eluted, and chromatographed on oligo(dT)-cellulose. (- - -) [^{3}H]Adenosine radioactivity; (—) [^{14}C]adenine radioactivity.

containing components have been removed by oligo(dT)-cellulose chromatography, was mixed with polysomes labeled with [^{14}C]adenine. The mixture was subjected to limited RNase digestion, phenol treatment, adsorption on Millipore, and fractionation on oligo(dT)-cellulose. Figure 5 shows that the adenosine-labeled fragments failed to interact in any stable fashion with the poly(A)-containing fragments derived from polysomes.

Dissociation of the Poly(A)-Containing Structure. The thermal denaturation profile of the poly(A)-containing structure is shown in Figure 6. Dissociation, as judged by loss of capacity to bind to Millipore, occurs within a relatively narrow range of temperatures. The profile appears to be biphasic, but the bulk of the material is released from the structure in the lower temperature range. The heat treatment was done under ionic conditions (10 m*M* Tris-HCl (pH 7.6)–10 m*M* KCl–1 m*M* $MgCl_2$) that strongly favor base pairing (Boedtker, 1960). Thus, the T_m of about 50° indicates that the interaction between the fragments is rather weak.

Möller and Boedtker (1962) have suggested that heating may cause chain breaks in RNA. In order to eliminate the possibility of chain breakage during the heat treatment, we

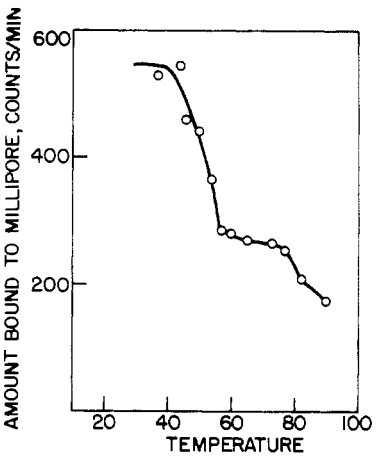

FIGURE 6: Thermal denaturation of the poly(A)-containing structure. Fraction C, which originated from polysomes labeled with [^{3}H]uridine and [^{3}H]hypoxanthine, was brought to 10 m*M* Tris-HCl (pH 7.6)–10 m*M* KCl–1 m*M* $MgCl_2$. Aliquots were heated for 5 min at various temperatures, quickly cooled, and adsorbed to Millipore filters in the presence of 10 m*M* Tris-HCl (pH 7.6)–500 m*M* KCl–1 m*M* $MgCl_2$.

Table IV: Effect of Various Dissociating Treatments on Binding to Millipore of RNA Fragments Eluted from Oligo(dT)-Cellulose Column.[a]

Buffer			Formamide (75%)	Ribonuclease (15 μg/ml)
0°	70°	90°	37°	37°
604	329	201	218	143

[a] Fraction C, prepared as in Figure 6, was precipitated with ethanol and 0.1 *M* NaCl at −20°. Precipitates were dissolved in 10 m*M* Tris-HCl (pH 7.6)–10 m*M* KCl–1 m*M* $MgCl_2$ prior to denaturation. Aliquots were treated for 5 min as described and diluted in ice-cold high ionic strength buffer for binding to Millipore filters. For the formamide treatment, aliquots in Tris–KCl–$MgCl_2$ were diluted with 3 volumes of formamide. Ribonuclease treatment was carried out in appropriate buffer (see Materials and Methods).

examined the effect of formamide, an agent that disrupts noncovalently attached polynucleotide chains (Ts'o et al., 1962), on the integrity of the poly(A)-containing structure. Formamide treatment releases the same amount of radioactivity from the structure as heating to 90° (Table IV). The results suggest that the main effect of heating is to dissociate noncovalently attached polynucleotides from the poly(A)-containing structure.

Some RNase-sensitive material remains associated with the poly(A) after heating at 90° (Table IV). This non-poly(A) material is apparently covalently linked to poly(A) sequences in the structure. Since poly(A) is found only at the 3′ terminus of mammalian mRNA (Mendecki et al., 1972; Molloy et al., 1972a; Sheldon et al., 1972b; Nakazato et al., 1973) the remaining material is presumably located in the region of the molecule adjacent to the poly(A) segment.

Characteristics of the Dissociated Fragments. The electrophoretic mobility of the fragments dissociated from the poly(A)-containing structure by heating at 70° is shown in Figure 7. It can be seen that the fragments are highly heterogeneous with respect to size, and that the majority of the fragments are considerably larger than the poly(A) segment.

Polyacrylamide gel electrophoresis of the poly(A)-con-

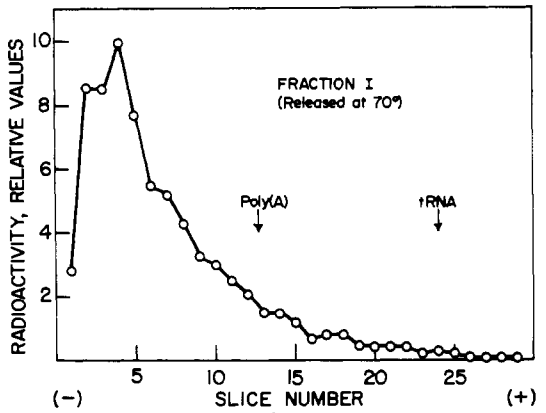

FIGURE 7: Polyacrylamide gel electrophoresis of RNA fragments dissociated from the poly(A)-containing structure at 70°. Polysomal RNA labeled with [^{3}H]uridine and [^{3}H]hypoxanthine was digested with RNase under mild conditions. The poly(A)-containing structure was isolated by two cycles of oligo(dT)-cellulose chromatography and heated to 70°, and bound (fraction II) and unbound (fraction I) materials were separated. An aliquot of pooled fraction I was mixed with [^{14}C]tRNA and electrophoresis was conducted in 7.5% polyacrylamide gels at 1 mA cm^{-1} for 150 min. Arrows indicate position of [^{14}C]tRNA, and of free poly(A) run separately.

Table V: Annealing of Heat-Dissociated Fragment to Poly(A)-Containing Fragment.[a]

		Additions during Annealing			
				Fraction II	
Poly(U)	Total (cpm)	None	Poly(A)	5 μl	20 μl
None	416	28	98	90	150
0.5 μg		34	40		64

[a] Adenosine-labeled fragments released from the poly(A)-containing structure by heat dissociation (fraction I_{70}) were incubated with unlabeled poly(A)-containing components (fraction II) or with 0.14 μg of synthetic poly(A), in the presence or absence of poly(U). Incubation mixtures were heated at 90° for 5 min in the presence of 10 m*M* Tris-HCl (pH 7.6)–10 m*M* KCl–1 m*M* $MgCl_2$, quickly cooled, and brought to 0.3 *M* NaCl–0.03 *M* sodium citrate (pH 7.0). Incubations were carried out at 50° for 24 hr and incubation mixtures filtered through Millipore as described in Table I. Values represent radioactivity retained on the filters.

taining fragments remaining after the heat treatment is shown in Figure 8. Complete RNase digestion of this fragment reveals a relatively homogeneous poly(A) component with the usual mobility (Figure 8B). The undigested poly(A)-containing fragment is far more heterogeneous, with most of the components larger than the free poly(A) (Figure 8A). In order to examine the distribution of the poly(A) segments in the various components, aliquots of the material extracted from gel slices were digested exhaustively with RNase. This shows that much of the poly(A) is associated with additional sequences which cause it to migrate more slowly (Figure 8A). Some of the poly(A) appears to be free of additional sequences. Thus it appears that the fragment resistant to dissociation at 70° consists of the poly(A) segment associated with varying lengths of the adjacent polynucleotide sequence in mRNA.

Annealing of Dissociated Fragments. Annealing experiments were carried out in order to determine whether the interaction between the components in the poly(A)-containing structure is due to an intrinsic property of the polynucleotide sequences involved. The fragments devoid of poly(A), isolated from adenosine-labeled polysomes, were

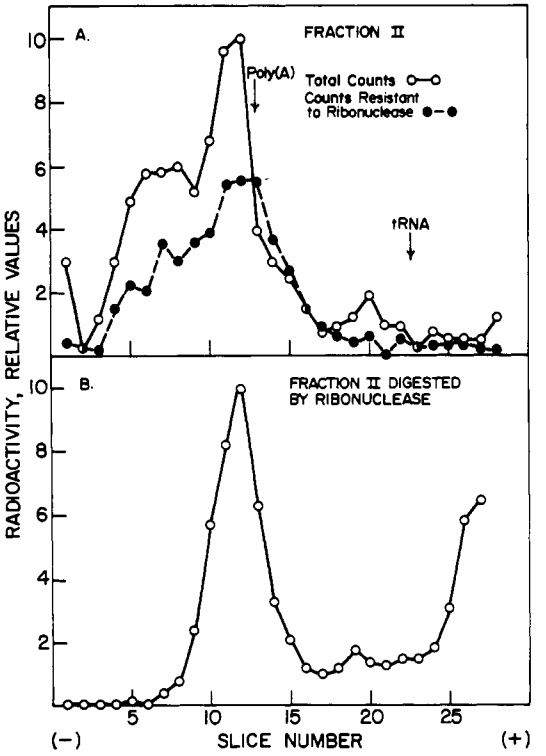

FIGURE 8: Polyacrylamide gel electrophoresis of poly(A)-containing fragments treated at 70°. Fraction II was prepared from polysomes digested with RNase. Electrophoresis was carried out as in Figure 7. (A) Electrophoretic profile of the total and RNase-resistant material of fraction II. Material was eluted from gel slices, and acid-insoluble material was counted with and without prior RNase digestion (1 μg/ml) for 30 min at 30° in 50 m*M* Tris-HCl (pH 7.6)–50 m*M* KCl–1 m*M* $MgCl_2$. (—) Total fraction II radioactivity; (- - -) RNase-resistant radioactivity. Arrow indicates position of [^{14}C]tRNA. (B) Electrophoretic profile of RNase-digested fraction II. Fraction II was digested with 1 μg/ml of RNase in 50 m*M* Tris-HCl (pH 7.6)–50 m*M* KCl–1 m*M* $MgCl_2$ for 30 min at 30° prior to electrophoresis.

incubated in the presence of the poly(A)-containing components derived from unlabeled polysomes. A substantial amount of reassociation took place, as judged by recovery of the capacity to bind to Millipore filters (Table V). The amount of reassociation increased with the quantity of poly(A)-containing segment present during the annealing. The interaction appears to involve the poly(A) sequence, since synthetic poly(A) can replace the polysomal fragment. The inhibitory effect of poly(U) further supports the involvement of the poly(A) sequence in the reassociation.

Discussion

The findings of the present study suggest a role for the poly(A) sequence in determining the configuration of mRNA in polysomes. A portion of the poly(A) segments appears to be associated with another polyribonucleotide sequence. This latter sequence could also be part of the mRNA molecule, although there is not direct evidence on this point. The polynucleotide segment that interacts with the poly(A) is not part of rRNA, since it becomes labeled in the presence of levels of actinomycin D that selectively block rRNA synthesis. Further work will be required to clarify this point. In the meantime, it is tempting to speculate on some implications of the possibility that a portion of the mRNA folds back to interact with the poly(A) segment in the course of translation. The sequence involved would have to be in a noncoding region of the molecule. It could

possibly be located near the 5′-terminus, in front of the initiation site. This would generate a circular structure, with the termination and initiation sites in close proximity. A circular mRNA structure has been proposed previously to explain some functional characteristics of isolated mammalian polysomes (Phillips, 1965; Baglioni et al., 1969). Circular polysomal structures have been detected in electron micrographs (Mathias et al., 1964). The physiological significance of such an arrangement is not obvious at the present.

The nature of the interaction with poly(A) appears to be through complementary base pairing. This is suggested by the annealing experiments (Table V). The relatively narrow range of melting temperatures indicates that the base-paired sequences are similar in most of the mRNA molecules. Such complementary base pairing would imply that a U-rich sequence must be present in mRNA. This would not need to be a pure poly(U) sequence, since polynucleotides can form partially base-paired structures in which the unmatched bases are looped out from the helix (Fresco et al., 1960). Sequences rich in U have been detected in heterogeneous nuclear RNA (Molloy et al., 1972b; Korwek et al., 1974), and apparently also in cytoplasmic RNA (M. Edmonds, personal communication).

The isolated polynucleotide segments associated with the poly(A) are unlikely to be part of a structure resistant to RNase. They are rather large and highly heterogeneous in size. They must have been generated by limited random fragmentation of polysomes or polysomal RNA. More extensive fragmentation destroys most of this material, and exhaustive RNase digestion does not appear to leave measurable amounts of uridine-labeled material associated with the poly(A). A more critical search for a residual U-rich component after extensive RNase digestion seems in order. It is likely that the length of the polynucleotide sequence that interacts with the poly(A) is relatively small. A significant portion of the poly(A) in the structure remains available for interaction with oligo(dT), poly(U), or cellulose nitrate. The occurrence on the poly(A) segment of protein (Kwan and Brawerman, 1972; Blobel, 1973) as well as the double-stranded structure described here indicates that this region of the polysome may be quite complex.

The validity of the present findings depends entirely on the effectiveness of the control experiments designed to verify that no artificial interactions were generated during the manipulations. For instance the poly(A) segment and a sequence complementary to it on the same RNA molecule could readily anneal during or after deproteinization. Any such segments that were not in duplex structure in the polysomes would have become separated by the RNase treatment. Since annealing between segments on different molecules should be much slower, the isolation of the structure from RNase-treated polysomes provides support for its occurrence in the polysomes. The possibility of less defined interactions during deproteinization of the polysome fragments is made unlikely by the experiment in which labeled RNA free of poly(A) was added to unlabeled polysomes prior to fragmentation (Figure 5). Our results point out the importance of carrying out deproteinization under conditions that do not cause denaturation in nucleic acid when the secondary structure of RNA components is to be investigated.

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Products Obtained after in Vitro Reaction of 7,12-Dimethylbenz[*a*]anthracene 5,6-Oxide with Nucleic Acids†

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ABSTRACT: Several lines of evidence suggest that oxide derivatives of carcinogenic polycyclic hydrocarbons are the reactive intermediates for in vivo binding to cellular nucleic acids. In the present study the covalent binding of 7,12-dimethylbenz[*a*]anthracene 5,6-oxide to synthetic homopolymers and nucleic acids in aqueous–acetone solutions has been investigated. Poly(G) was found to be the most reactive nucleic acid and underwent approximately 7–10% modification. Alkaline hydrolysis of the poly(G)–dimethylbenzanthracene conjugate yielded chromatographically distinct polycyclic hydrocarbon-modified nucleotides which were further characterized by spectral analyses and enzymatic and chemical degradation. When the oxide was allowed to react with GMP or dGMP, at least two products were obtained in about 1% yield. Acid hydrolysis of the dGMP–dimethylbenzanthracene conjugates liberated the corresponding guanine–dimethylbenzanthracene products. Mass spectral analysis of the modified bases provided direct evidence that we had obtained covalent binding of the polycyclic hydrocarbon to guanine. The mass spectral cleavage patterns suggest that one of these products is a hydroxydihydro derivative of dimethylbenzanthracene bound to guanine and the other is a dimethylbenzanthracene–guanine conjugate. Additional structural aspects of these guanine derivatives are discussed.

The covalent binding of carcinogenic polycyclic aromatic hydrocarbons[1] (PAHs) to nucleic acids in the intact animal (Brookes and Lawley, 1964) and in cell culture (Baird et al., 1973; Duncan et al., 1969) is well established and it seems likely that this interaction is essential for the carcinogenic process (Miller, 1970). Since the parent compounds are not themselves chemically reactive, they must undergo metabolic conversion in vivo to activated forms capable of chemical interaction with the target molecules. The aryl hydrocarbon hydroxylase system, associated with the microsomal fraction of cells, is responsible for the conversion of PAHs into a number of oxidized derivatives (Jerina and Daly, 1974; Sims et al., 1973). This group of enzymes is inducible by a variety of substrates and probably plays a key role in both the detoxification and activation of carcinogenic PAHs (Gelboin, 1969a,b; Borden et al., 1973). Several laboratories (Gelboin, 1969a; Grover and Sims, 1969; Borden et al., 1973; Pietropaolo and Weinstein, 1975) have shown that incubation of tritium-labeled PAHs in a subcellular system containing rat liver microsomes and NADPH leads to covalent binding of the PAH to nucleic acids, but the precise reaction mechanism and structures of the derivatives have not been elucidated.

In 1950 Boyland proposed that arene oxides might be the primary intermediates in the oxidative metabolism of PAHs to hydroxylated derivatives and suggested that the transient and chemically reactive oxides may be the proximate carcinogens. Subsequent studies have established arene oxides as intermediates in the microsomal oxidation of PAHs and the K-region oxides of DMBA (Keysell et al., 1973) and other PAHs (Sims et al., 1971; Grover et al., 1972) have indeed been detected after incubation of the parent hydrocar-

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[1] Abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; DMBA oxide, 7,12-dimethylbenz[*a*]anthracene 5,6-oxide; PAH, polycyclic aromatic hydrocarbon; poly(G)-DMBA, GMP-DMBA, and dGMP-DMBA are the nucleic acid products obtained after treating poly(G), GMP, and dGMP, respectively, with DMBA oxide (the DMBA abbreviation in these cases does not necessarily indicate that the parent hydrocarbon itself is present in the product); λ_{max}, wavelength of maximal absorbance in the ultraviolet (uv) spectrum; $A_{310}/A_{\lambda_{max}}$ ratio of absorbance at 310 nm to that at λ_{max}; MS, mass spectrum.