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In Vitro Processing of Intervening Sequences in the Precursors of Messenger Ribonucleic Acid for Adenovirus 2 Deoxyribonucleic Acid Binding Protein†

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ABSTRACT: Early region 2 of the adenovirus 2 genome (map positions 61–75) specifies two polyadenylated nuclear RNAs (28 S and 23 S) that appear to be precursors of the 20S cytoplasmic mRNA [Goldenberg, C. J., & Raskas, H. J. (1979) *Cell 16*, 131–138]. Isolated nuclei were used to study the processing of region 2 RNA in vitro. Cultures infected with adenovirus 2 were pulse labeled with [^{3}H]uridine early in infection. Nuclei were purified from the labeled cultures and then incubated in vitro. Incubations were performed in the absence or presence of a cytosol extract isolated in hypotonic conditions from uninfected cells. During in vitro incubation, nuclear 28S and 23S region 2 RNAs were converted to 20S molecules. In the presence of the cytosol, the conversion to 20S RNA molecules was greatly accelerated and almost completed after 10 min of incubation. The conversion was quantitative, and the resulting 20S RNAs were stable for at least 30 additional min. The cytosol activity was concentration dependent and temperature sensitive. A cytosol prepared in isotonic conditions was inactive, suggesting that the processing activity was nuclear in origin. Early region 2 RNAs processed in vitro were analyzed for splicing patterns. RNA was purified by using a preparative membrane hybridization–selection procedure. The selected RNAs (20 S) were fractionated by size and then rehybridized to a series of DNA fragments. The data demonstrated (1) that the processed 20S RNAs had lost sequences as expected on the basis of known splicing patterns and (2) that these 20S RNAs contained leader sequences spliced to the body of the RNA molecule.

At least four regions of the adenovirus 2 genome are expressed at early times [see review by Flint (1977)]. Each of these early regions has at least one independent promoter for the initiation of transcription (Craig & Raskas, 1976; Berk & Sharp, 1977; Evans et al., 1977). We have been studying the processing of RNAs synthesized by early region 2. The early mRNA from this region is specified by DNA sequences within map positions 61–75 (Flint & Sharp, 1974; Pettersson et al., 1976); the mRNA is copied from the strand transcribed in the leftward direction (Sharp et al., 1974). The cytoplasmic mRNA consists of 70 nucleotides from positions 75 to 74.6 spliced to 170 nucleotides from 68.8 to 68.3 and 1700 nucleotides from 66.3 to 61.6 (Kitchingman et al., 1977; Berk & Sharp, 1978; see Figure 1). The polypeptide product of this mRNA is a 72 000-dalton DNA binding protein (Ginsberg et al., 1974; Grodziker et al., 1974; Van der Vliet et al., 1975; Lewis et al., 1976).

We have characterized the structures of three polyadenylated nuclear RNAs that appear to be precursors of the cytoplasmic mRNA for the DNA binding protein (Craig & Raskas, 1976; Goldenberg & Raskas, 1979) (Figure 1). All the intervening DNA sequences of the gene for the 72K DNA binding protein are contained in the largest (28 S) mRNA precursor. Apparently these sequences are removed sequentially to generate first a 23S intermediate and then a 20S nuclear RNA that is transported to the cytoplasm as mature mRNA. Our studies of the region 2 nuclear RNAs provide support for a processing model in which mRNA is generated by RNA cleavage followed by splicing (Klessig, 1977; Berget et al., 1977; Chow et al., 1977). The internal cleavage of mRNA precursors requires activities that recognize specific sequences; the resulting termini must then be ligated to form a covalent bond. Recently, splicing of yeast tRNA in vitro has been reported. The tRNA precursor contains a 15-nucleotide intervening sequence that is not present in the mature molecules (Valenzuela et al., 1978). A cell-free enzymatic activity which can remove the intervening sequences and religate the ends has been identified (Knapp et al., 1978, 1979; Peebles et al., 1979).

Several years ago, our laboratory described an in vitro nuclear system in which adenovirus high molecular weight RNA was converted to the size of mRNAs (Brunner & Raskas, 1972). These studies preceded the availability of specific DNA fragments, and therefore it was not possible to analyze pre-

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cursors of specific mRNAs. Subsequently, adenovirus DNA fragments were used to analyze in vitro size changes of late nuclear RNAs having sequence relationships to fiber mRNA (Zimmer et al., 1978); the size changes were compatible with processing events, but RNA splicing was not examined. For the present study, we utilized information gained from the in vivo experiments in which we identified nuclear precursors of region 2 mRNA. Here we demonstrate the in vitro processing of region 2 precursors contained in nuclei isolated early in infection. Similar size changes for region 2 RNAs were also observed recently by Blanchard et al. (1978). Our study demonstrates that the processing has several properties of an enzymatic activity. Moreover, it yields RNA with at least one splice point similar to that found in the mature mRNA.

Materials and Methods

Cell Cultures, Viral Infections, and Preparation of Nuclei. Maintenance of KB cell suspension cultures, procedures for adenovirus 2 infections, and labeling conditions with [^{3}H]-uridine were performed as previously described (Craig et al., 1975). All infections were performed in the presence of cytosine arabinoside to block viral DNA replication. Nuclei were isolated from cultures harvested early in infection (6 h). Nuclei were prepared by treatment with 0.2% Nonidet P-40 in isotonic buffer (30 mM Tris-HCl,[1] pH 7.6, 150 mM NaCl, and 1.5 mM $MgCl_2$) (Goldenberg & Raskas, 1979).

In Vitro Incubations. Reactions for in vitro processing were performed with 5×10^7 nuclei/mL in an incubation buffer containing 30 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 0.5 mM each of GTP, CTP, UTP, and ATP, 15% glycerol, 10 mM creatine phosphate, and 100 μg/mL creatine phosphokinase. Incubations were at 37 °C for the times indicated in the figure legends; reactions were terminated by the addition of RNA extraction buffer [see below; Holmes & Bonner (1973)]. For some experiments, nuclei were incubated in the presence of cytosol. The cytoplasmic extract (cytosol) was isolated by resuspending 1×10^8 KB cells in 1 mL of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, and 2 mM DTT). The cells were allowed to swell for 10 min at 4 °C, followed by lysis with a Dounce homogenizer. The lysate was centrifuged at 800*g* for 5 min, and the supernatant was clarified by centrifugation at 15000*g* for 10 min. Fresh cytosol was prepared for each experiment.

Nucleic Acid Procedures. Purification of polyadenylated nuclear RNAs, isolation of virus specific RNAs, fractionation by electrophoresis in polyacrylamide–formamide gels, and RNA–DNA hybridization conditions have been described previously (Craig & Raskas, 1974a,b; 1976). Conditions for restriction enzyme digestion and purification of DNA fragments were reported by Goldenberg & Raskas (1979).

Results

The three size classes (28 S, 23 S, and 20 S) of polyadenylated nuclear RNAs transcribed from the l strand of early region 2 are shown in Figure 1. The intervening DNA sequences of the mature mRNA are contained in the 28S RNA complementary to map positions 75–61.6. The nuclear 23S and 20S RNAs lack the sequences that are derived from map positions 74.5–68.8. The nuclear 20S RNA, like the mature mRNA, also lacks sequences from the second splice region, map positions 68.3–66.3. In addition to the structural data, the results of metabolic experiments are also consistent

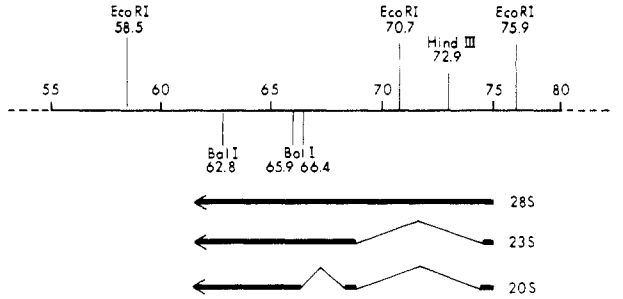

FIGURE 1: Transcription and restriction enzyme cleavage maps of early region 2 of the adenovirus 2 genome. Relevant sites of endo R·*Eco*RI, endo R·*Bal*I, and endo R·*Hin*dIII have been reported previously (Mulder et al., 1974; McGrogan & Raskas, 1978; R. Gelinas, 1979, personal communication). The map positions indicate the relative distance of the cleavage site from the left end of the genome. The solid bars represent the structures of the nuclear RNAs (Goldenberg & Raskas, 1979). The cytoplasmic mRNA has the structure of the 20S species (Kitchingman et al., 1977; Berk & Sharp, 1978). The arrows indicate the direction of transcription. The caret-shaped symbols indicate sequences covalently joined by splicing (Goldenberg & Raskas, 1979).

with the proposed precursor–product relationships; in continuous-label experiments, the 28S and 23S RNAs become uniformly labeled more rapidly than molecules the size of the cytoplasmic mRNA (Goldenberg & Raskas, 1979).

RNA Processing during Incubation of Nuclei in Vitro. Cultures were labeled with [^{3}H]uridine for 30 min beginning 5 h after adenovirus 2 infection to obtain nuclei that would serve as substrates for studies of region 2 RNA processing in vitro. The purified nuclei were incubated at 37 °C in buffer containing ribose triphosphates. Samples were withdrawn for RNA analysis at various times during incubation. The purified polyadenylated nuclear RNA from each sample was subjected to electrophoresis in formamide–polyacrylamide gels. After electrophoresis, the RNA in each gel slice was eluted and hybridized to reveal the profile of region 2 sequences. For these hybridizations, we utilized a DNA probe (map positions 62.8–65.9) which anneals only the region 2 RNAs of interest. Previous studies which used large DNA fragments from this region of the genome had documented the existence of early r strand transcripts in the nucleus which are not related to the early region 2 mRNA (Goldenberg & Raskas, 1979). These r strand molecules are excluded by the use of the 62.8–65.9 probe.

The results of a typical experiment are shown in the left three panels of Figure 2. Prior to incubation of the prelabeled nuclei, the major species is the nuclear 28S RNA. After 30 min of incubation, approximately two-thirds of the RNA has been converted to a 20S species which comigrates with the cytoplasmic mRNA. It should be noted that the hybridization probe (62.8–65.9) facilitates quantitative comparisons. The leftward transcripts from map positions 62.8–65.9 are represented uniformly in all three of the nuclear species (see Figure 1). Therefore, the counts per minute hybridized after RNase treatment are a direct reflection of the relative numbers of molecules in each size class.

Enhanced Processing of Nuclear RNAs in the Presence of Cytosol Extract. The conversion of 28S and 23S RNAs to 20S molecules was incomplete in some experiments as shown in the left three panels of Figure 2. It has previously been shown that during preparation of nuclei in hypotonic buffers certain nuclear components may leak into the "cytosol" extract (Wu, 1978). Therefore, we examined the possibility that a cytosol might contain factors that would enhance nuclear activities. Cytosol extracts prepared in this manner were added

[1] Abbreviations used: DTT, dithiothreitol; ATP, CTP, GTP, and UTP, adenosine, cytidine, guanosine, and uridine 5′-triphosphates; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

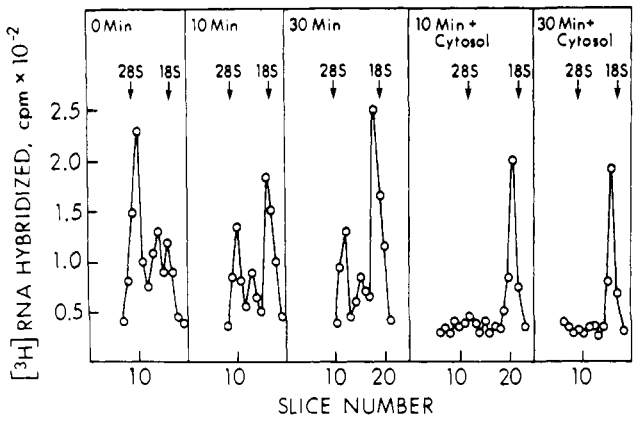

FIGURE 2: In vitro processing of nuclear RNA precursors of the mRNA for 72K DNA binding protein. Cultures infected with adenovirus 2 were labeled with [^{3}H]uridine for 10 min (200 μCi/mL, 5×10^6 cells/mL) beginning 5.9 h after infection. Nuclei (5×10^8) were then isolated in isotonic conditions as described under Materials and Methods. Aliquots of 1×10^8 nuclei were incubated in 2 mL of incubation buffer at 37 °C. Two samples were incubated in the presence of 0.5 mL of cytosol purified from 5×10^7 uninfected cells (right two panels; see Materials and Methods). Polyadenylated nuclear RNAs were purified, denatured, and subjected to electrophoresis. Eluted RNAs from each gel slice were hybridized to approximately 1-μg equivalents (the amount of the DNA fragment that would be derived from 1 μg of whole genome DNA) of the 62.8–65.9 DNA fragment. For the nuclear RNAs incubated in the absence of cytosol, the polyadenylated RNAs subjected to electrophoresis were from approximately 200 μg of total nuclear RNA. The nuclear RNAs incubated in the presence of cytosol utilized the ^{3}H-polyadenylated RNAs from approximately 100 μg of total nuclear RNA.

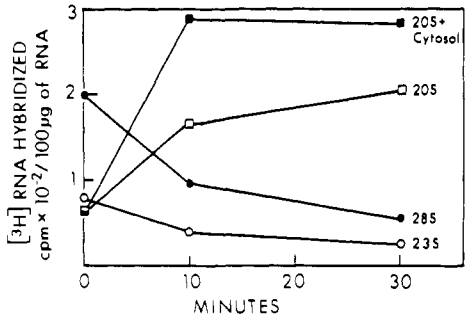

FIGURE 3: Kinetics of in vitro processing of early region 2 RNA. The radioactivity in each peak of Figure 2 was summed, the background subtracted, and the counts per minute normalized to the amount of RNA loaded on each gel. The results are presented as ^{3}H cpm hybridized to a 62.8–65.9 DNA fragment per 100 μg of total RNA. (●) 28S RNA; (○) 23S RNA and (□) 20S RNA from nuclei incubated in the absence of cytosol; and (■) 20S RNA from nuclei incubated in the presence of cytosol.

to the nuclear incubation mixtures. The results are shown in the two right panels of Figure 2. In the presence of cytosol, the conversion to 20S molecules was greatly accelerated and was completed in 10 min. The resulting 20S RNAs were stable for at least 20 min more, indicating that no extensive degradation occurs after the processing is complete.

The kinetics of the RNA conversions were quantitated and are illustrated in Figure 3. As suggested above, conversion in the presence of cytosol is more rapid and extensive. Moreover, the conversions are quantitative; the counts per minute in 20S RNA at the end of incubation were equal to the sum of the 28S, 23S, and 20S counts per minute at time zero. Cytosols from uninfected and infected cells were equally effective (data not shown). In contrast, cytosol prepared in isotonic buffer did not enhance processing activity (not shown), as would be expected if the active cytosol components were originally nuclear in origin and leaked in hypotonic buffers.

Table I: Inactivation of the Processing Activity of Cytosol Extract by Preincubation at Different Temperatures[a]

nuclei incubated with cytosol preincubated at	28 S		23 S		20 S	
	cpm	% of total cpm	cpm	% of total cpm	cpm	% of total cpm
40 °C	1050	56	340	17	510	26
50 °C	1060	57	330	17	460	25
0 °C					1250	80
nuclei not incubated	1196	66	240	13	376	21

[a] Nuclei were prepared from infected cultures labeled with [^{3}H]-uridine, [^{3}H]guanosine, and [^{3}H]adenosine as described in Figure 5. Aliquots of 1×10^8 nuclei were incubated for 15 min at 37 °C in the presence of 500 μL of cytosol isolated from 5×10^7 uninfected cells. The cytosol extract, in 10% glycerol, was preincubated for 15 min at the indicated temperatures. Polyadenylated nuclear RNA was purified, subjected to electrophoresis, eluted, and hybridized to 1-μg equivalents of the 62.8–65.9 DNA fragment. The radioactivities in 28S, 23S, and 20S RNAs were summed, the backgrounds subtracted, and the counts per minute normalized to the amount of RNA loaded on each gel.

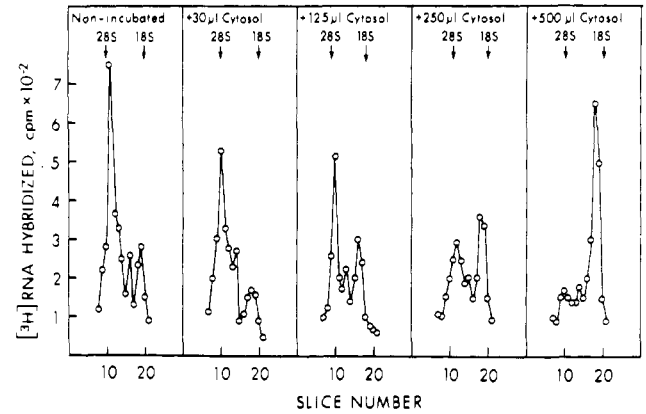

FIGURE 4: Effect of different concentrations of cytosol on the processing of region 2 nuclear RNA precursors. Nuclei were isolated from infected cultures labeled with [^{3}H]uridine as described in Figure 2. Aliquots of 1×10^8 nuclei were incubated for 15 min at 37 °C in the presence of different amounts of cytosol extract isolated from uninfected cells. The total reaction volume was 2.0 mL. Polyadenylated nuclear RNA was purified, denatured, and subjected to electrophoresis. Eluted RNAs from each gel slice were hybridized to approximately 1-μg equivalents of 62.8–65.9 DNA.

The cytosol activity which enhances in vitro processing of adenovirus RNAs is temperature labile. Preincubation of the cytosol at 40 °C for 15 min inactivated the processing activity (Table I). To investigate if in vitro processing depends on the concentration of cytosol, nuclei labeled in vivo with [^{3}H]uridine were incubated in vitro for 15 min in the presence of different concentrations of extract. Figure 4 shows that the rate of conversion of nuclear RNA precursors to 20S RNA increased with increasing concentrations of cytosol. The optimum rate of region 2 RNA processing was attained when extract from 5×10^7 cells was incubated with 1×10^8 nuclei (Figure 4, right panel).

Splicing of Region 2 20S RNA Processed in Vitro. To test whether the 20S RNA processed in vitro is spliced as the region 2 mRNA, we analyzed the structure of polyadenylated RNA isolated from nuclei incubated in the presence of cytosol extract. The region 2 RNAs were purified by using a preparative membrane hybridization and elution procedure (McGrogan et al., 1979). RNAs selected by the 62.8–65.9 DNA fragment were eluted and then subjected to electrophoresis in formamide gels (Figure 5, left panel). As expected, this fragment selected RNAs which migrated as a single 20S

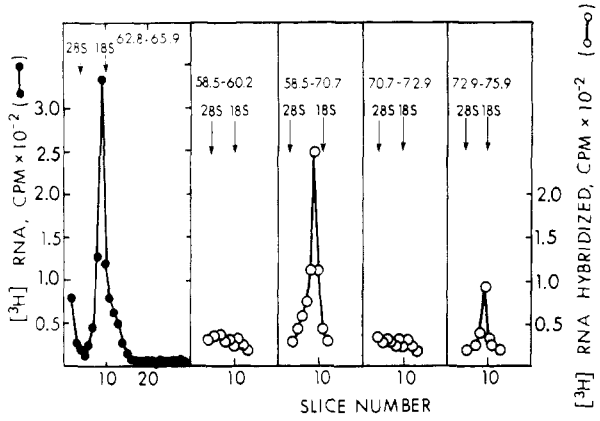

FIGURE 5: Splicing pattern of the 20S region 2 RNA processed in vitro in the presence of cytosol. Infected cultures (5×10^8 cells) were labeled with [^{3}H]uridine, [^{3}H]guanosine, and [^{3}H]adenosine (200 μCi/mL each, 5×10^6 cells/mL) for 10 min. Nuclei were isolated and incubated in vitro at 37 °C for 15 min in the presence of cytosol isolated from 2×10^8 uninfected cells. Polyadenylated nuclear RNA (200×10^6 cpm) was purified, and hybridization selections were performed with 62.8–65.9 DNA (Goldenberg & Raskas, 1979). The selected RNAs (85 000 cpm) were subjected to electrophoresis in formamide gels. After electrophoresis, the RNA was eluted from each gel slice; 5-μL aliquots were counted directly (●, left panel). Equal aliquots (125 μL) of the eluted RNAs were then annealed to 1-μg equivalents of the indicated DNA fragments (○, four right panels).

peak. The selected 20S RNA was then rehybridized to a series of fragments from adjacent regions of the genome. The 20S RNA hybridized efficiently to 58.5–70.7 and 72.9–75.9 DNA fragments. However, it did not hybridize to the 70.7–72.9 DNA fragment. This fragment contains part of the intervening sequences (map positions 74.5–68.8) of the mature mRNA (see Figure 1). The selectivity of the selection and hybridization method was confirmed by the absence of hybridization to the 58.5–60.2 DNA fragment, a fragment unrelated to region 2. Thus, incubation in vitro in the presence of cytosol extract produced 20S RNA that contained at least one major splice of the mature mRNA.

Discussion

The many observations of RNA splicing in cellular and viral mRNAs provide compelling evidence that this phenomenon is a fundamental biological process. For this reason, it is an important current goal to obtain in vitro splicing systems which are amenable to biochemical analysis. In vivo studies of the early mRNA specified by adenovirus early region 2 provided several observations that encouraged the utilization of this system to study processing in vitro. (1) There is a single mRNA product of 1920 nucleotides; given the complex biochemical steps that must be required for RNA splicing, it should be advantageous to analyze an independently regulated region with only one gene and one gene product. (2) The region 2 mRNA is 20 S in size and consists of three spliced segments, 1700, 150, and 70 nucleotides; presumably, a minimum of two splicing events is required to generate the mRNA. (3) Nuclei contain two polyadenylated RNAs that are putative precursors of the mRNA. These RNAs are larger (28 S and 23 S), are derived from the same strand as the mRNA (Craig et al., 1977), and have labeling kinetics compatible with their being precursors (Goldenberg & Raskas, 1979). These precursors offer the opportunity to study sequential steps in RNA processing. The structure of the two nuclear RNAs is such that they appear to have 5′ and 3′ termini common to those of the mRNA and in addition contain intervening sequences.

For initial studies of in vitro region 2 processing, we chose to utilize isolated nuclei. Earlier experiments performed in our laboratory had provided evidence that reproducible size changes were obtained when late nuclei were incubated in vitro (Brunner & Raskas, 1972). More specifically, RNAs with sequences related to fiber mRNA were cleaved in vitro to gene products the size of fiber mRNA (Zimmer et al., 1978). The experimental design utilized here is straightforward. Infected cultures are labeled with [^{3}H]uridine in vivo, and isolated nuclei are purified. Subsequent incubation of isolated nuclei in vitro yielded a partial conversion of region 2 28S and 23S RNAs to molecules the size of mRNA, 20 S. Since the conversion was incomplete, we developed a procedure for accelerating and completing the size change. A cytosol was used to supplement the incubation mixture. The cytosol preparations were effective only if prepared from cells swollen in hypotonic buffer. Isotonic preparations were inactive. From these observations, we infer that the cytosol activity is in fact nuclear in origin and leaks from the nuclear compartment during cell fractionation. A similar finding has been made with respect to eukaryotic RNA polymerases III and II activities (Wu, 1978; Weil et al., 1979a,b). For future studies and purification, the cytosol may be a valuable source of splicing activity.

The current studies are significant because they provide evidence that splicing indeed occurs in vitro. Simple observations of size changes might be misleading (Blanchard et al., 1978); RNAs might diminish in size by removal of 5′ terminal sequences rather than by genuine splicing. Our data do not allow the conclusion that the in vitro splicing mimics precisely the in vivo events. However, they demonstrate in 20S RNA the linkage of sequences from two widely separated regions, as occurs in mature mRNA from region 2. It should be noted that splicing requires at least two distinct functions, cleavage and ligation. These two functions may be performed by different molecules. Here we have shown that these functions can be performed on RNA in isolated nuclei. Future studies will seek to characterize the nature of the cytosol splicing activity and to determine if this activity can act on naked RNA with fidelity.

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Polypeptides of Nonpolyribosomal Messenger Ribonucleoprotein Complexes of Sea Urchin Eggs†

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ABSTRACT: RNA competent in directing protein synthesis is sequestered in unfertilized sea urchin eggs as translationally quiescent, nonpolyribosomal, messenger ribonucleoprotein complexes (mRNPs). Following fertilization, these mRNPs are derepressed and actively translated, presumably due to changes in the mRNA-associated proteins and their interaction with the mRNA. We have isolated poly(A)-containing egg mRNPs free of contaminating monoribosomes and ribosomal subunits by chromatography on oligo(dT)–cellulose and identified their constituent proteins. Egg mRNPs isolated by using near physiological ionic conditions have 15–20 major proteins, most of which are in the molecular weight range of 40 000–100 000, and ~15–23 minor proteins in the 22 000–190 000 molecular weight range. The association of the proteins with poly(A)-containing mRNA is indicated by their greatly reduced retention on oligo(dT)–cellulose after pretreatment of the crude mRNP fraction with saturating amounts of poly(uridylic acid). Three of the proteins present in poly(A)-containing mRNPs from eggs, with molecular weights of 48 000, 67 000, and 140 000, were not detected in poly(A)-containing mRNPs derived from polyribosomes of hatched blastula-stage embryos. In addition, stoichiometric differences were found between some of the proteins associated with the two types of mRNP. The potential regulatory role of these proteins is discussed.

As a consequence of the number and complexity of the components involved in translation, there are numerous potential mechanisms for regulating the total rate of protein synthesis as well as the synthesis of specific proteins. Since eucaryotic mRNA exists in association with specific proteins from the time of its nuclear synthesis until its involvement in polyribosome formation [reviewed by Spirin (1979)], and probably until its degradation, it is likely that these proteins affect the utilization of mRNA. Generally less than 15 proteins have been found in both messenger ribonucleoprotein complexes (mRNPs)[1] that are free in the cytosol and mRNPs derived from polyribosomes (Cardelli & Pitot, 1977; Grubman

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[1] Abbreviations used: mRNPs, messenger ribonucleoprotein complexes; hnRNPs, heterogeneous nuclear ribonucleoprotein complexes; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; A_{260nm} unit, the amount of material per 1 mL having an absorbance of 1 at 260 nm in a 1-cm path length.

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