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Ribonucleic Acid Precursors Are Associated with the Chick Oviduct Nuclear Matrix†

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ABSTRACT: Nuclear matrix was prepared by sequential treatment of oviduct nuclei with Triton X-100, DNase I, and 2 M NaCl. Published procedures were modified such that as many steps as possible were performed at −20 °C to minimize endogenous ribonuclease activity. Examination of electron micrographs confirmed the isolation of intact nuclear matrix structures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the proteins in these structures showed an absence of histones and an enrichment of certain nonhistone proteins. RNA was isolated from the nuclear matrix preparations and subjected to denaturing gel electrophoresis. Gels were analyzed by ethidium bromide staining and by hybridization of Northern blots to cloned DNA probes for ovalbumin, ovomucoid, 5.8S ribosomal RNA, and U1 RNA. All of the precursors to ovalbumin and ovomucoid mRNAs (including various splicing intermediates) and all of the precursors to ribosomal RNA were associated exclusively with the nuclear matrix fraction. By contrast, mature ovalbumin and ovomucoid mRNAs were distributed between matrix and nonmatrix fractions. These observations were further supported by quantitative hybridization analysis of the RNA in nuclear and matrix fractions. It was found that less than 50% of the mature message of intact nuclei was recovered in the matrix, while most significantly, over 95% of the mRNA precursors remained associated with the matrix. Finally, mature ribosomal RNAs and virtually all of the small nuclear RNAs (including U1 RNA) were also distributed between matrix and nonmatrix fractions. Our results suggest that all precursor RNAs (be they precursors to mRNA or rRNA) are exclusively associated with the nuclear matrix and support the notion that the nuclear matrix may be the structural site for RNA processing within the nuclei of eucaryotic cells.

Intervening sequences have been observed in nuclear RNA transcripts of animals, plants, and lower eucaryotes and in RNA transcripts encoded by eucaryotic viruses (Abelson, 1979; Roop et al., 1978). Although not all eucaryotic RNA transcripts are interrupted by intervening sequences [such as those for U1 RNA, histones, or interferon (Roop et al., 1981; Schaffner et al., 1978; Nagata et al., 1980)], removal of intervening sequences by RNA splicing appears to be a general mechanism by which the majority of functional mRNAs are manufactured in eucaryotic cells.

Earlier studies have demonstrated that a large fraction of

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heterogeneous nuclear RNA (hnRNA) is tenaciously associated with a subnuclear structure that has been termed the nuclear matrix (Faiferman & Pogo, 1975; Miller et al., 1978a; Herman et al., 1978; Long et al., 1979; Van Eekelen & Van Venrooij, 1981). The nuclear matrix consists of a fibrillar network, made up mostly of protein that remains after nuclei have been digested with DNase I and extracted with high salt and detergent (Berezney & Coffey, 1974; Hodge et al., 1977; Kaufmann et al., 1981). hnRNA does not appear to be an integral component of the matrix since RNase digestion releases most of the hnRNA sequences without altering the matrix structure (Kaufmann et al., 1981). Kinetic evidence suggests that newly synthesized RNA transcipts rapidly associate with the nuclear matrix (Faiferman & Pogo, 1975; Miller et al., 1978a). This study has attempted to further define the subset of hnRNA associated with the nuclear matrix by determining the subnuclear location of RNA precursors to ovalbumin and ovomucoid mRNA.

Ovomucoid and ovalbumin mRNAs are two of the RNAs that are synthesized in large amounts in oviducts of chicks that have been injected with estrogen. Nuclei isolated from such oviducts have been shown to contain ovalbumin and ovomucoid RNA at levels that comprise 0.4% and 0.12% of the total nuclear RNA, respectively (Tsai et al., 1978; Roop et al., 1978). The genes for ovalbumin (Dugaiczyk et al., 1978, 1979) and ovomucoid (Catterall et al., 1979; Lai et al., 1979) each have seven intervening sequences. Analysis of the nuclear forms of ovalbumin or ovomucoid RNAs by Northern gel blots has revealed the existence of multiple high molecular weight RNA precursors that include a putative primary transcript and many splicing intermediates (Roop et al., 1978; Nordstrom et al., 1979). The structure of the high molecular weight forms of the ovalbumin and ovomucoid RNAs has been established by hybridization to cloned gene probes (Roop et al., 1978, 1980; Nordstrom et al., 1979) and by electron microscopy (Tsai et al., 1980), and their role as precursors to mRNA has been established by pulse-label and chase experiments (Tsai et al., 1980).

Experimental Procedures

Materials

All solutions were filtered through 0.22-μm membrane filters. DNase I (Boehringer) was purified from contaminating RNase by chromatography over agarose-5′-[(*p*-aminophenyl)phosphoryl]uridine 2′(3′)-phosphate (Miles) according to the procedure of Maxwell et al. (1977). [^{32}P]dCTP and [^{32}P]dTTP (2000–3000 Ci/mmol) were purchased from Amersham. The −20 °C bath was maintained by a mixture of salt and ice in the ratio of 1:3.

Methods

Cloned DNA Probes. The plasmid pOV230 contains a full-length ovalbumin cDNA insert, lacking only 12 nucleotides at the 5′ end (McReynolds et al., 1977). OV2.4 contains a portion of the 5′ end of the natural ovalbumin gene which consists mainly of intervening sequences, only 7% of which represent structural sequence (Roop et al., 1978). The majority of the chicken ovomucoid gene, except 120 nucleotides at the 3′ end, is contained in the plasmid pOM14 (Lai et al., 1979). The 5.88 ribosomal DNA probe is a cloned cDNA to chicken 5.8S ribosomal RNA (p5.8S) (P. Kristo et al., unpublished results), and the U1 RNA probe is a cloned cDNA to chicken U1 RNA (pU1) (Roop et al., 1981).

Procedure at −20 °C for the Isolation of Chick Oviduct Nuclear Matrix. Oviducts from chicks stimulated with diethylstilbesterol (Tsai et al., 1975) were removed into ice-cold buffer A [40% (v/v) glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.6] and weighed. The tissue was cleaned of blood vessels and connective tissue, placed in fresh ice-cold buffer A (6 mL/g of tissue), minced with scissors, and homogenized in a tissuemizer at 4 °C 3 times for 15 s each. The homogenate was filtered through cheesecloth and organza and placed immediately into a −20 °C ice–salt bath. Crude nuclei were then collected by centrifugation at 4000*g* for 10 min at −20 °C. The crude nuclear pellet was suspended in ice-cold buffer B (2.2 M sucrose, 50 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$) at 6 mL/g of tissue. The suspension was homogenized by three strokes in a Potter–Elvehjem homogenizer; subsequently, the nuclei were purified by centrifugation at 55000*g* for 90 min at 0 °C.

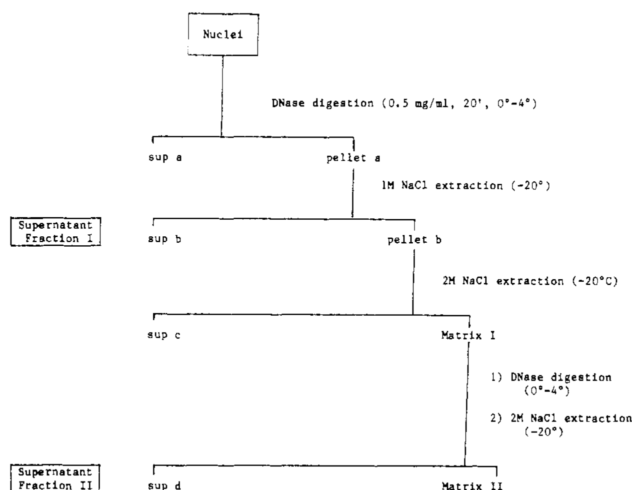


FIGURE 1: Summary of the procedure for the isolation of chick oviduct nuclear matrix. All steps in the fractionation procedure were performed at −20 °C, except for certain individual steps that were performed at 0–4 °C, as indicated.

The steps for the isolation of nuclear matrix are outlined in Figure 1. All steps, except the 20-min DNase I digestions, were performed at −20 °C. Centrifugations at −20 °C were at 4000*g* for 10 min. The purified nuclei were suspended in −20 °C buffer A (3 mL/g), homogenized by three strokes in a Potter–Elvehjem homogenizer, and recollected by centrifugation. The nuclei were washed once with buffer A plus 0.5% Triton and then with buffer A again. After resuspension in buffer A, the nuclei were mixed with an equal volume of DNase I (1.0 mg/mL in 10 mM Tris-HCl, pH 7.4, and 3 mM $MgCl_2$) and incubated for 20 min at 0–4 °C with occasional agitation. Three volumes of −20 °C buffer A was then added and the mixture centrifuged. The pellet was suspended in −20 °C buffer A (1.5 mL/g) and mixed with an equal volume of buffer C [2.0 M NaCl, 40% (v/v) glycerol, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4]. The mixture was incubated at −20 °C for 10 min and then centrifuged. The resultant "pellet" is termed nuclear matrix I. The supernatants from the last three centrifugations were combined and termed supernatant fraction I. Nuclear matrix I was suspended in buffer A (0.4 mL/g), incubated at −20 °C for 10 min, and finally centrifuged. The resulting pellet which has been through two DNase digestions and two salt extractions is termed nuclear matrix II, and the resulting supernatants are termed supernatant fraction II.

RNA Extraction, Northern Gel Blots, and RNA–DNA Hybridization. Fractions were adjusted to contain 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (NaDodSO$_4$), 4 M urea, and 0.2 M NaCl. RNA

was extracted with an equal volume of phenol mixture (phenol–chloroform–isoamyl alcohol, 25:24:1 v/v) as described previously (Roop et al., 1978). RNA was extracted directly from supernatant fractions with an equal volume of phenol mixture. Samples were treated with DNase I at a concentration of 40 μg/mL for 20 min at room temperature, reextracted with the phenol mixture, and reprecipitated with ethanol before application to the gel. Electrophoresis of RNA in methyl mercuric hydroxide agarose gels, transfer to DBM paper, and hybridization to nick-translated DNA probes were carried out as previously described (Roop et al., 1978; Nordstrom et al., 1979). RNA–DNA hybridizations were performed under conditions of RNA excess as described previously (Roop et al., 1978).

Electrophoresis of snRNA. After extraction as described above, the RNA was dissolved in 70% formamide, boiled for 5 min, and then loaded onto 10% acrylamide gels [acrylamide–bis(acrylamide), 30:1] containing 7 M urea as described by Maniatis et al. (1975). The gel was then stained with ethidium bromide and photographed.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Nuclei and nuclear matrix fractions were pelleted by centrifugation for 5 min in a microfuge and dissolved in loading buffer (0.0625 M Tris-HCl, pH 6.8, 3% $NaDodSO_4$, 5% 2-mecaptoethanol, and 10% glycerol). Soluble fractions were precipitated with 5% trichloroacetic acid (4 °C, 15 min) and collected by centrifugation at 16000*g* for 30 min. The precipitated protein was washed 3 times with diethyl ether and then dissolved in loading buffer. Samples were loaded onto acrylamide slab gels [4.5% stacking gel, pH 6.8; 12.5% separating gel, pH 8.8; acrylamide–bis(acrylamide), 37.5:1] and electrophoresed according to Laemmli (1970). Following electrophoresis, gels were stained with Coomassie Blue.

Chemical Analyses. RNA and DNA present in the various fractions were separated by the Schmidt & Thannhauser (1945) procedure. The amount of DNA was determined by the diphenylamine reaction as modified by Burton (1956) using calf thymus DNA as a standard. The amount of RNA was determined by the orcinol procedure with yeast RNA as a standard. Protein concentration was measured by the method of Bradford (1976), with BSA as a standard.

Electron Microscopy. Samples were fixed with 3% glutaraldehyde and 1% OsO_4 and then stained en bloc with saturated uranyl acetate in 50% ethanol at 4 °C (overnight for nuclear fractions and 36 h for matrix fractions). The samples were dehydrated, infiltrated with Spurr's plastic, and embedded. The samples were poststained for 30 min with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate for 10 min. Thin sections were examined on a Siemens–Elimskop 102 electron microscope.

Preparation of ^{3}H-Labeled 60S Ribosomal Subunits and [^{3}H]hnRNP Particles. ^{3}H-Labeled 60S ribosomal particles were prepared by incubating 100 mL of HeLa cells (at a cell density of 2×10^5 cells/mL) overnight in the presence of 1 mCi of [^{3}H]uridine at 37 °C. The cells were centrifuged at 1000 rpm for 15 min and washed 2 times with 15 mL of cold Tris–saline (150 mM NaCl, 10 mM Tris, pH 7.4, and 1.5 mM $MgCl_2$) and made 1% in NP40. This suspension was vortexed briefly and then centrifuged at 4 °C at 2000 rpm for 2 min. The supernatant was collected, adjusted to 10 mM EDTA, and layered onto a 15–40% sucrose gradient. This was centrifuged in a SW40 rotor at 35 000 rpm for 6 h. The location of the 60S subunit was identified by OD_{260} readings of fractions obtained from the gradient after centrifugation. [^{3}H]-hnRNP particles were obtained from HeLa cells, pretreated for 10 min with 0.04 μg/mL actinomycin D, and then incubated with [^{3}H]uridine for 30 min and treated as described by Pederson (1974).

Results

In a study designed to evaluate the association of a particular population of RNA molecules with the nuclear matrix, it is essential that the conditions used for matrix isolation do not permit the degradation of the associated RNA. Chick oviduct nuclear matrix was initially isolated by conventional techniques in which all steps were performed at 0–4 °C (Berezney & Coffey, 1974; Long et al., 1979). Steps included the isolation of oviduct nuclei by centrifugation through neutral sucrose solutions, treatment with Triton X-100, digestion with RNase-free DNase I, and extraction with concentrated salt solutions. When the RNA in the nuclear matrix fractions prepared by this procedure was extracted and analyzed by Northern gel blots, only highly degraded RNA molecules were evident (data not shown). These results showed that isolation of nuclear matrix from chick oviduct by conventional procedures was not suitable for the analysis of specific mRNA molecules and their precursors. Modification by inclusion of ribonuclease inhibitors, aurintricarboxylic acid (Hallick et al., 1977), or vanadylribonucleoside complex (Berger & Birkenmeier, 1979) was unsuccessful. Although they effectively blocked RNA degradation, these agents also prevented the isolation of a suitable nuclear matrix. For example, analysis of fractions prepared in the presence of either inhibitor by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed no differences in the proteins present in intact nuclei and nuclei that had been subjected to DNase digestion and salt extraction (data not shown).

The successful method that was developed involves the use of low temperature (−20 °C) as the primary means by which endogenous ribonuclease activity was minimized (Figure 1). This method was originally developed by Schibler & Weber (1974) to isolate undegraded RNA from *Xenopus* larval liver. As soon as possible after its homogenization, oviduct tissue was chilled to −20 °C in an ice–salt bath. Buffers contained 40% (v/v) glycerol to prevent freezing. All subsequent steps in the procedure, except two, were performed completely at −20 °C. One step that was necessary to perform at 0–4 °C was centrifugation of crude nuclei through 2.2 M sucrose. Inclusion of this step was found to be necessary to purify the nuclei and minimize the extent of cytoplasmic contamination (as evidenced by electron microscopy). The second step performed at 0–4 °C was the DNase I digestion, which was allowed to proceed only for brief periods (20 min) at high enzyme concentration (0.5 mg/mL).

The nuclear matrices prepared by this modified procedure closely resemble those isolated by using conventional methods as judged by several criteria: electron microscopy, chemical composition, and $NaDodSO_4$–polyacrylamide gel electrophoresis. Electron micrographs of nuclei and nuclear matrices generated by the modified procedure are depicted in Figure 2. Matrix I is the result of a single DNase I digestion and high salt extraction, whereas matrix II is the result of two consecutive DNase I and high salt treatments. The matrix fractions have been depleted of virtually all of their chromatin and much of their other nuclear material but retain evidence of internal particles and fibrils that are organized into a network bounded by a peripheral nuclear lamina. Remnants of the nucleolar region within the internal matrix structure are also evident. The nuclear matrix structures isolated from oviduct by our modified procedure, thus, closely resemble those prepared by others (Kaufmann et al., 1981).

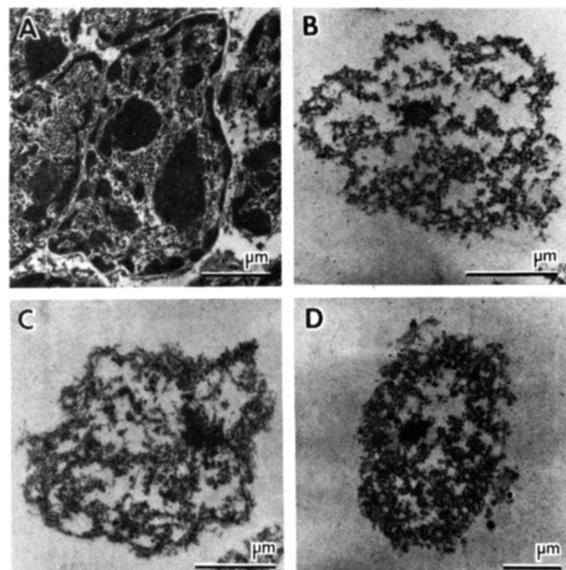


FIGURE 2: Electron microscopy of chick oviduct nuclei and nuclear matrix. (A) Nuclei; (B) nuclear matrix I; (C and D) nuclear matrix II.

Table I: Composition of Oviduct Matrix[a]

	RNA[c]	DNA	protein
nuclei[b]	100%	100%	100%
matrix I	15-20%	3-4%	20%
matrix II	9%	1%	7%

[a] Values represent the average of four matrix preparations. [b] Initial RNA/DNA ratio = 0.48. [c] RNA recovery (matrix I + supernatants a, b, and c) = 80-100%.

An analysis of the chemical content of the nuclear matrix from oviduct is shown in Table I. The majority of the protein, DNA, and RNA has been removed from the nuclei, the DNA almost to completion. As expected, the mass of the resultant matrix structure consists mostly (68%) of proteins. Due to the care to minimize ribonuclease activity, a significant amount of RNA is also retained and comprises 25% of the mass of the matrix.

Analysis of the nuclear matrix proteins by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3) reveals that the matrix as prepared from oviduct is virtually devoid of core histones (the quartet of bands from 14K to 17K), histone H1 (31K), and many of the nonhistone polypeptides present in intact nuclei (for example, the bands at 33K, 39K, and 82K). The polypeptide pattern of the final nuclear matrix fraction is complex, however. Of the many bands that are evident, polypeptides having molecular weights of 37K, 44K, 54K, 68K, and 70K and several that are greater than 100 000 are rather prominent. The prominence of these bands on $NaDodSO_4$ gels is characteristic of many nuclear matrices prepared from a variety of sources (Long et al., 1979; Berezney, 1980; Peters & Comings, 1980; Kaufmann et al., 1981). The 68K- and 70K-dalton polypeptides are similar in size to the polypeptides that are typically characteristic of peripheral nuclear lamina (Gerace et al., 1978; Krone et al., 1978).

Analysis of Specific RNAs and Their Association with the Nuclear Matrix. (A) Ovalbumin mRNA Precursors. For determination of the intranuclear location of ovalbumin mRNA precursors, oviduct nuclei were purified through 2.2 M sucrose, washed with 0.5% Triton X-100, and then fractionated into nuclear matrix and supernatant fractions as described under Experimental Procedures. RNA was extracted from each fraction and analyzed by hybridization of

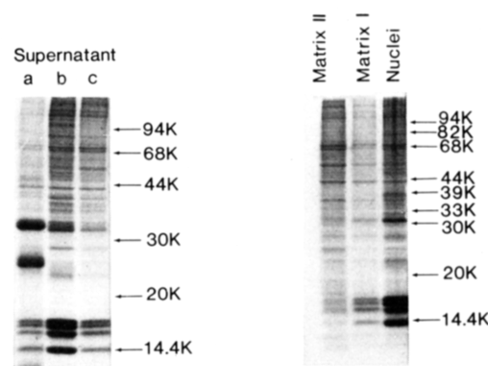


FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of nuclear matrix proteins. Chick oviduct nuclear fractions were prepared as outlined in Figure 1. Each lane was loaded with 20 μg of protein. Molecular weight markers were the following: phosphorylase *b* (94K); bovine serum albumin (67K); ovalbumin (43K); carbonic anhydrase (30K); soybean trypsin inhibitor (20.1K); and α-lactalbumin (14.4K).

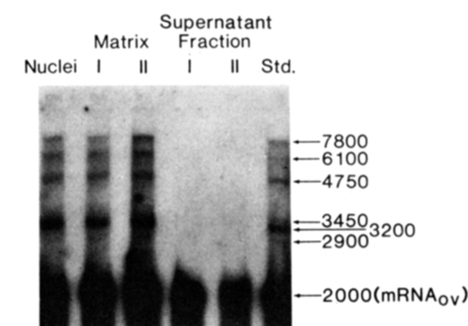


FIGURE 4: Association of ovalbumin mRNA precursors with the oviduct nuclear matrix. RNA was extracted from each of the nuclear subfractions prepared from 40 g of oviduct tissue as outlined in Figure 1. The total amount of RNA recovered in equivalent aliquots of each fraction (i.e., as if each fraction was exclusively derived from 40 g of oviduct tissue) was nuclei (3200 μg), matrix I (480 μg), matrix II (224 μg), supernatant fraction I (2688 μg), and supernatant fraction II (224 μg). Approximately equal amounts of RNA from each fraction (15 μg) were then applied to the gel, except that the amount of RNA applied from supernatant fraction I was 60 μg. After loading, samples were subjected to electrophoresis, blotting, and hybridization to the cloned ovalbumin cDNA probe (pOV230). A 20-μg sample of total oviduct nuclear RNA, isolated under totally denaturing conditions, was used as the standard. The nucleotide sizes of the ovalbumin mRNA precursors and ovalbumin mRNA ($mRNA_{ov}$) are indicated.

Northern gel blots to ^{32}P-labeled cloned ovalbumin DNA probes. Figure 4 shows that all of the ovalbumin mRNA precursors, including the unspliced precursor that has a size of 7800 nucleotides, were found associated with the nuclear matrix fraction.

On the basis of the intensity of the ovalbumin RNA bands, all of the ovalbumin mRNA precursors were significantly enriched in the matrix II fraction when compared to matrix I and especially when compared to total oviduct nuclear RNA. No ovalbumin mRNA precursors were detected in the supernatant fractions, even when the amount of RNA loaded on the gel was increased 4-fold. In addition, treatments of nuclear matrix II with additional DNase I and high salt solutions failed to release any of the ovalbumin precursor RNAs. The association of the ovalbumin RNA precursors with the nuclear matrix from oviduct was resistant to 1% Triton X-100 and 50 mM EDTA. Thus far, we have found that matrix associated RNAs are released only by agents ($NaDodSO_4$ and

Table II: Recovery of $mRNA_{OV}$ and $RNA_{2.4}$ in Matrix Fractions

fraction	RNA sequences recovered (%)	
	$cDNA_{OV}$	$RNA_{2.4}$
nuclei	100	100
matrix II	46	96

urea) that disrupt the entire matrix structure. It appears, therefore, that ovalbumin mRNA precursors are tightly and exclusively associated with the nuclear matrix fraction.

Quantitative hybridization analysis of matrix-associated ovalbumin RNA precursors was carried out to further document this finding. For these experiments, a cloned DNA fragment of the ovalbumin gene (OV2.4) that contains 93% intervening sequence was used as a hybridization probe. Results shown in Table II indicate that the nuclear matrix II fraction retains over 95% of the ovalbumin intervening RNA sequences that are found in the whole nuclei. We can conclude, therefore, that ovalbumin mRNA precursors are exclusively associated with the oviduct nuclear matrix. On the basis of the recovery of RNA in the nuclear matrix II (Table I), we calculate that ovalbumin RNA precursors in the nuclear matrix are enriched about 10-fold.

(*B*) *Ovalbumin mRNA*. Figure 4 also demonstrates that mature ovalbumin mRNA (the large band centered at 2000 nucleotides in length) is also associated with the nuclear matrix structure. During the preparation of the matrix, however, a large portion of the mature ovalbumin mRNA that is present in whole oviduct nuclei was released into the supernatant fractions. Quantitative hybridization experiments using cloned ovalbumin cDNA as the probe for ovalbumin structural RNA sequences indicated that less than 50% of the mature ovalbumin mRNA found associated with oviduct nuclei was retained in the nuclear matrix. Further treatment of nuclear matrix II failed to release additional ovalbumin mRNA molecules. Therefore, the ovalbumin mRNA that is associated with the matrix is as tightly bound as the ovalbumin mRNA precursors.

(*C*) *Ovomucoid mRNA and Its Precursors*. Analysis of the oviduct nuclear matrix for ovomucoid RNAs is shown in Figure 5. All of the ovomucoid mRNA precursors, including the yet to be spliced precursor that has a size of 5450 nucleotides, are found associated with the nuclear matrix fraction. As was observed with the ovalbumin RNA precursors, all of the ovomucoid mRNA precursors were highly enriched in the matrix II fraction and none were released into the supernatant fractions. Mature ovomucoid mRNA was also found associated with the matrix, but a large portion of the ovomucoid mRNA molecules present in whole nuclei was released into the supernatant fractions during matrix isolation. Thus, ovomucoid mRNA and its precursors are associated with the oviduct nuclear matrix in the same way as ovalbumin mRNA and its precursors.

(*D*) *Ribosomal RNAs and Their Precursors*. The association of all of the ovalbumin and ovomucoid RNA precursors and a significant portion of their mature mRNAs with the nuclear matrix contrasted sharply with the distribution of the majority of the nuclear RNA. Over 90% of the RNA is released into the supernatant fractions during the preparation of the matrix (Table I). When analyzed on gels and stained with ethidium bromide, the predominant RNA species that are released into the supernatant fractions are 28S and 18S ribosomal RNAs and a group of small RNAs (unresolved on agarose gels) that include 5.8S, 5S, and transfer RNAs and other small nuclear RNAs. Although the vast majority of these stable RNAs have been released, a significant amount

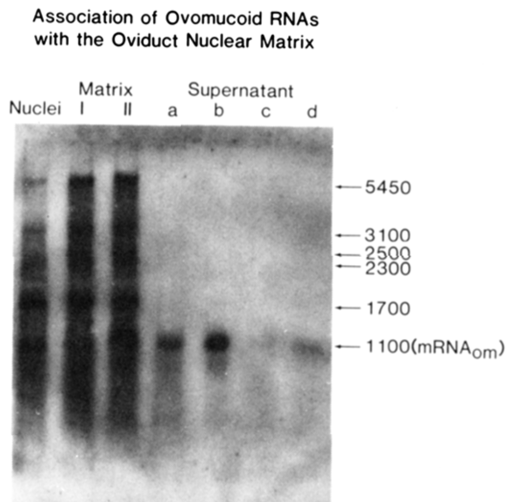


FIGURE 5: Association of ovomucoid mRNA precursors with the oviduct nuclear matrix. RNA from each of the nuclear subfractions was analyzed by Northern gel blots exactly as described in Figure 4, except that hybridization was to the ovomucoid DNA probe (pOM14). The nucleotide sizes of the ovomucoid mRNA precursors and ovomucoid mRNA ($mRNA_{om}$) are indicated.

remains associated with the oviduct nuclear matrix (Figure 6A). Analysis of the bands reveals the presence of 45S and 32S ribosomal RNA precursors in total nuclear fractions and in the nuclear matrix fractions, but not in the supernatant fractions. It appears, therefore, that ribosomal RNA precursors, like the mRNA precursors, are exclusively associated with the oviduct nuclear matrix.

To strengthen this conclusion, we used cloned cDNA to chick 5.8S ribosomal RNA as a specific hybridization probe to study the association of ribosomal precursor RNAs with the nuclear matrix. Analysis of total nuclear RNA with this probe as shown in Figure 6B reveals ribosomal RNA bands that were 11 000, 6000, and 160 nucleotides in length. These RNAs correspond to the 45S rRNA precursor, the 32S rRNA precursor, and the mature 5.8S rRNA, respectively. An additional RNA band of 1300 nucleotides was also detected with this probe. This RNA molecule was most likely generated by further processing of the 32S rRNA precursor by cleavage near its 5′ end (Lewin, 1980). The resulting 1300-nucleotide RNA probably consists, therefore, of 5.8S RNA sequences flanked by spacer RNA sequences. All three of the detected ribosomal RNA precursors were found to be associated with the nuclear matrix fraction (Figure 6B), and virtually none were found in the supernatant fractions. Mature 5.8S rRNA was also found to be associated with the matrix fraction, but the majority of it was released into the supernatant fractions. Thus, the oviduct nuclear matrix retains all of the ribosomal RNA precursors but retains only a small fraction of the mature (25S, 18S, 5.8S) ribosomal RNAs.

(*E*) *Small Nuclear RNAs*. It has been postulated that small nuclear RNAs (snRNAs) may serve as helpers in RNA splicing (Lerner et al., 1980; Rogers & Wall, 1980; Ting et al., 1981). Particular attention has been focused on U1 RNA with regard to the splicing of mRNA precursors. Previous reports have claimed that all (Miller et al., 1978b) or a specific few (U2 and 4.5 S; Zieve & Penman, 1976) of the snRNAs are found highly enriched in the nuclear matrix. In addition, it has been recently demonstrated that the snRNAs may be isolated in the form of small ribonucleoprotein particles (snRNPs) that are separable from hnRNPs (Lerner et al., 1980). For determination of the subnuclear location of oviduct snRNAs, oviduct nuclei were fractionated into matrix and

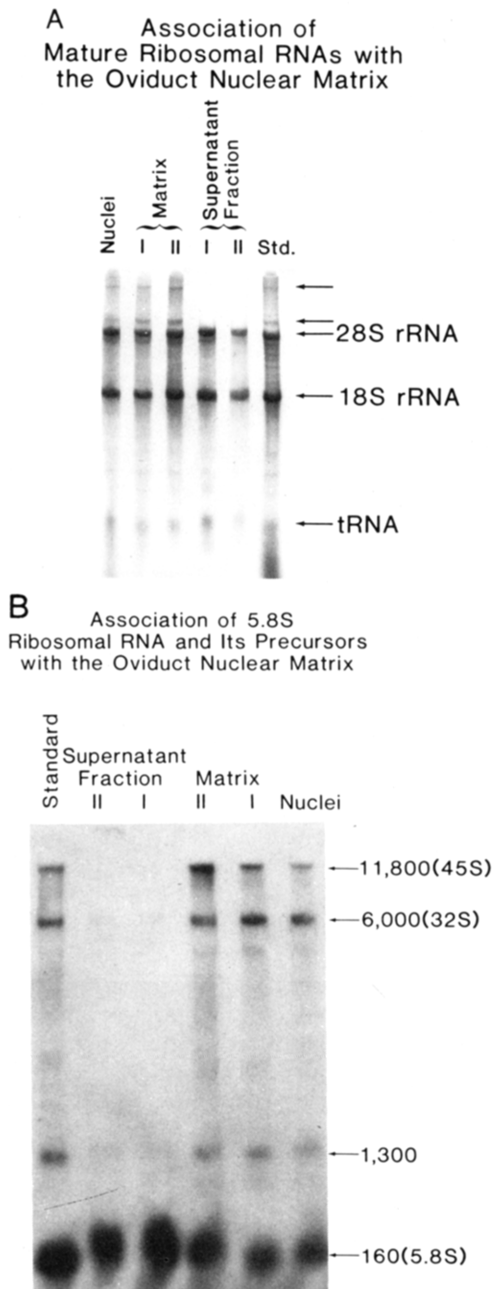
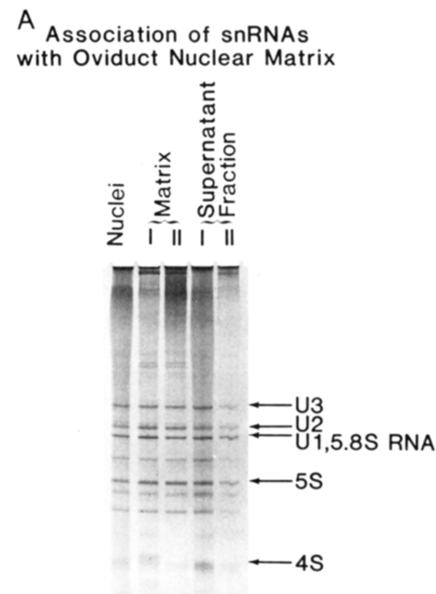


FIGURE 6: Association of ribosomal RNAs with the oviduct nuclear matrix. RNA from each of the nuclear subfractions was electrophoresed exactly as described in Figure 4. (A) Photograph of ethidium bromide stained gel. (B) Autoradiograph after Northern blot and hybridization to the chicken 5.8S ribosomal gene clone, p5.8S. The nucleotide sizes of mature 5.8S RNA and its precursors are indicated.



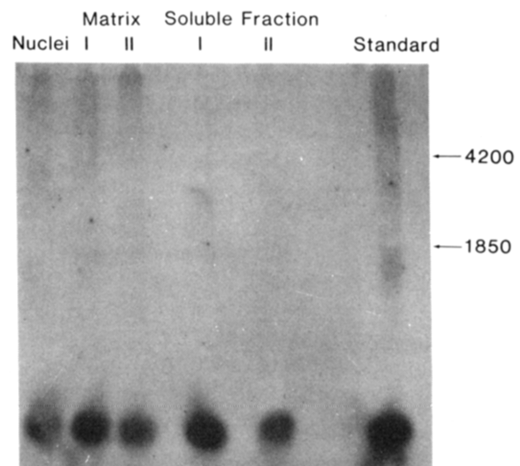


FIGURE 7: Analysis of snRNAs in oviduct nuclear subfractions. RNA from each of the nuclear subfractions was extracted, digested extensively with DNase I (RNase free), and loaded (in amounts identical with that of Figure 4) onto a polyacrylamide–urea or denaturing agarose gel. (A) Photograph of ethidium bromide stained polyacrylamide–urea gel. (B) Autoradiograph of agarose gel after Northern blot and hybridization to the cloned chicken U1 RNA gene, pU1.

supernatant fractions, and the presence of snRNAs was assayed by electrophoresis on denaturing polyacrylamide gels (Figure 7). Bands for the oviduct snRNAs were assigned according to the criteria developed by Roop et al. (1981). Most of the snRNAs that were equal to or less than U3 RNA in size were released into the supernatant fractions. However, a portion of these snRNAs remained in tight association with the nuclear matrix. No preferential enrichment in the matrix fraction of individual snRNAs, including U1 RNA, was observed except for two uncharacterized RNA species that were larger than U3 RNA. These RNAs (~290 and ~300 nucleotides in size) appear to be enriched similar to snRNAs K and M (Zieve, 1981) and are in the oviduct nuclear matrix. The larger of these two RNA molecules does not appear to be present in the supernatant fractions at all. Nevertheless, the major conclusion remains unchanged—none of the snRNAs that are believed to play a role in RNA splicing are enriched in the oviduct nuclear matrix.

To evaluate the association of U1 RNA with the oviduct nuclear matrix more rigorously, we used the cloned gene for chicken U1 RNA (Roop et al., 1981) as a specific hybridization probe (Figure 7B). Analysis of total nuclear RNA with this probe reveals a single U1 RNA band (the band is broad because an agarose gel was utilized for these blotting experiments). Although some U1 RNA remains associated with the nuclear matrix fraction, Figure 7B clearly shows the U1 RNA is released into the supernatant fractions during matrix isolation. Thus, in contrast to mRNA and rRNA precursors, U1 RNA exhibits no preferential association with the oviduct nuclear matrix.

In order to determine whether particular RNA molecules are specifically associated with the nuclear matrix, it is necessary to rule out the possibility that RNA molecules have become entrapped within or nonspecifically absorbed to the matrix structure. The following control experiments were

performed to monitor the nonspecific absorption of cytoplasmic or nuclear ribonucleoprotein particles to the oviduct nuclear matrix during its isolation.

^{3}H-Labeled 60S ribosomal particles from HeLa cells were added to isolated chick oviduct nuclei after they had been washed once with 0.5% Triton and once with buffer A as described under Experimental Procedures. Matrix was isolated as previously outlined, and the amount of label associated with the final matrix fraction was determined. Only 0.8% of the recovered radioactivity was found to contaminate the final matrix, and 99.2% was found in the resulting nonmatrix supernatants (97% of the original label was recovered). Therefore, the vast majority of the added ribosomal ribonucleoprotein particles was removed during the preparation of the nuclear matrix.

In the second experiment, ^{3}H-labeled hnRNP particles from HeLa cells were isolated and added to chick oviduct nuclei before the isolation of the matrix, as discussed above. In this case, 4% of the recovered radioactivity was found to contaminate the final matrix, and 96% was found in the resulting supernatant fractions. These results argue against the nonspecific interaction of hnRNP particles with the oviduct nuclear matrix.

Discussion

A method has been presented for the isolation of nuclear matrix that has intact RNA molecules associated with it. The cold-temperature procedure originally developed by Schibler & Weber (1974) was very useful in reducing the endogenous nuclease activities in oviduct tissue to an acceptable level. In addition, the cold temperature appeared to have minimized the extent of protein degradation as witnessed by the undegraded histone bands in the various fractions when examined in $NaDodSO_4$–polyacrylamide gel electrophoresis. Because original matrix isolation procedures called for the removal of 98% of the original nuclear RNA (Berezney & Coffey, 1974), we have examined the product of our procedure by several criteria. Electron microscopy, $NaDodSO_4$–polyacrylamide gel electrophoresis, and chemical composition have confirmed that oviduct matrix prepared by our procedure is comparable to that isolated by others. Therefore, this method should be applicable to the isolation and analysis of nuclear matrices from a variety of tissues.

The ability to isolate nuclear matrix with undegraded RNA allows us to closely examine the RNA associated with the matrix and to define a subset of hnRNA which preferentially associate with the matrix. Analysis of specific RNAs containing sequences homologous to the chicken ovomucoid, ovalbumin, 5.8S ribosomal RNA, and U1 RNA genes revealed a specific distribution of RNA between matrix and nonmatrix fractions. All precursor RNAs were found in the matrix fractions while their mature forms were found equally distributed between both matrix and nonmatrix fractions. Our data show that this association is not a random process and precursors are not preferentially found there because of their longer nucleotide sequence. First, our control experiment of adding back-labeled hnRNP particles showed no random association of the particles with the isolated matrices. Second, many of the mature forms are larger in size than some of the precursors. For example, ovalbumin mRNA (2000 nucleotides) is larger than some of the precursors to ovomucoid mRNA or 5.8S RNA. None of the smaller ovomucoid mRNA precursors are released from the matrix while a significant fraction of the mature ovalbumin mRNA is released. Another example is that most of the mature 28S (4970 nucleotides) and 18S (2076 nucleotides) ribosomal RNAs are released into the supernatant fractions, but a much smaller rRNA precursor (1300 nucleotides) remains associated with the nuclear matrix. Although our isolation involved several rigorous steps (centrifugation through 2.2 M sucrose and washing with Triton X-100), to obtain purified nuclei, the ability to remove mature forms of various RNAs suggested the possibility of cytoplasmic contamination. To control for this, we added labeled ribosomal 60S particles to the nuclei and demonstrated that isolated matrix showed little, if any, contamination with such exogenously added particles. Finally, the subnuclear distribution of snRNA indicates that the majority of snRNAs is not tightly associated with the nuclear matrix. This demonstrates unequivocally that not all nuclear RNAs are bound to the matrix structure. We conclude, therefore, that the association of RNAs with the nuclear matrix is dependent upon the sequence and structure of the individual RNA molecules.

The mode of association of the RNA precursors with the nuclear matrix is unknown as yet. An association of newly synthesized hnRNA with specific nuclear proteins was first discovered by Samarina et al. (1968). Analysis of the RNA component in these structures typically revealed the presence of degraded hnRNA sequences (Van Venrooij & Janssen, 1978). Recently, however, intact globin mRNA and its 15S precursor have been shown to be associated with ribonucleoprotein particles that sediment at 50–200 S that were prepared by sonication of Friend erythroleukemia cell nuclei (Pederson & Davis, 1980). Thus, intact eucaryotic mRNAs and their precursors can also be isolated in the form of RNPs. The relationship between nuclear RNPs and nuclear matrix is unclear. Sonication of nuclei probably causes disruption of the nuclear matrix (Berezney, 1980) and results in the release of RNPs that contain mRNAs and mRNA precursors. Both RNPs and nuclear matrix appear to contain the same RNA molecules, yet the nature of the RNA–protein interaction differs. Complete dissociation of RNPs occurs at 0.6 M salt (Beyer et al., 1977), whereas certain RNAs remain quantitatively bound when the nuclear matrix is exposed to salt as high at 2.0 M. This suggests that the RNAs in the nuclear matrix and in RNPs are bound by different proteins. However, recent experiments that involve RNA–protein cross-linking show that proteins involved in the binding of hnRNA to the nuclear matrix are similar to certain proteins that bind RNA in RNP core particles (Van Eekelen & Van Venrooij, 1981). These experiments show that the binding of hnRNA to the nuclear matrix from HeLa cells involves two proteins that are 41 500 and 43 000 daltons. These proteins appear to be similar to the group "C" proteins that are tightly associated with the RNA of HeLa cell RNP core particles (Beyer et al., 1977). It has also been shown that adenovirus nuclear RNAs that are bound to the HeLa cell nuclear matrix are associated with the same two proteins (Mariman et al., 1982). Thus, although their structure and resistance to dissociation by salt differ, hnRNA appears to interact with the nuclear matrix and RNP particles through a common set of proteins.

snRNAs have been implicated as helpers in RNA splicing by virtue of the fact that they exhibit sequence complementarity to the splice junctions found in RNA precursors. In the oviduct, the majority of each species of snRNA was not found to be tightly associated with the nuclear matrix, in contrast to the close association of RNA presursors, and this indicates that most snRNAs are not members of a complex that consists of RNA precursors and nuclear matrix proteins. This is consistent with the recent findings that snRNAs are found in separable snRNP particles that contain proteins that differ

from those that are characteristically found in hnRNP particles (Lerner et al., 1980; Lerner et al., 1981).

The association of RNA precursors with the chick oviduct nuclear matrix is most consistent with the hypothesis that the matrix is the structural site within the nucleus for RNA processing. In agreement with this, Mariman et al. (1982) have reported that precursors and mature mRNAs encoded by the late region of the adenovirus genome are associated with the nuclear matrix of HeLa cells, and Ross et al. (1982) have shown that globin RNA and its precursors are associated with the chicken erythroblast nuclear matrix. In addition, it has previously been shown that precursors to ribosomal RNA are found to be associated with the nuclear matrix of *Tetrahymena pyriformis* (Herlan et al., 1979). It was reported that the extent of association of the ribosomal RNA precursors to the matrix decreased as the processing of the molecules proceeded. This differs from our findings in that we observed that all the ribosomal precursors, regardless of their size or extent of processing, are exclusively associated with the nuclear matrix fraction.

In conclusion, we report the preferential association of a particular population of hnRNA molecules, RNA precursors, with the chick oviduct nuclear matrix. The association of these molecules with the nuclear matrix strongly supports the proposal that the matrix is the site within the nucleus for RNA processing. Preliminary studies suggest that nonspecific ribonuclease activity in oviduct nuclear matrix upon incubation at 22 °C is fairly low. Thus, it may be possible to begin testing whether the precursor RNAs that are in association with the nuclear matrix might serve as viable substrates for in vitro RNA processing reactions.

Acknowledgments

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Acid–Base Equilibrium of the Schiff Base in Bacteriorhodopsin†

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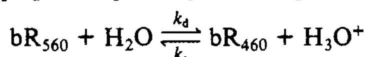
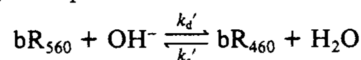
ABSTRACT: Aqueous suspensions of dark-adapted bacteriorhodopsin (bR_{560}) in the purple membrane of *Halobacterium halobium* are exposed to rapid jumps to high pH. Optical and resonance Raman measurements are carried out by using flow and stationary methods. Above pH $\simeq$11.5 bR_{560} starts to be reversibly converted to a species absorbing at 460 nm (bR_{460}) characterized by an unprotonated Schiff base chromophore. Above pH $\simeq$13.0 bleaching takes place, first reversibly and subsequently irreversibly, to a species absorbing around 365 nm (bR_{365}). This process competes with the formation of bR_{460}. The pK_a corresponding to the equilibrium



$$bR_{560} + H_2O \underset{k_a}{\overset{k_d}{\rightleftharpoons}} bR_{460} + H_3O^+$$

is determined as 13.3 ± 0.3. The value of the corresponding association rate constant determined from the reverse jumps (from pH 12.67 to pH 10 and 9.2) is $k_a = (3.5 \pm 0.5) \times 10^{11}$ M^{-1} s^{-1}. Thus, starting with bR at pH 12.67 the reprotonation process is diffusion controlled as observed for homogeneous acid–base equilibria. The observed rate of dissociation when jumping from pH 6.5 to 12–13 is slower than that predicted by including the equilibrium



$$bR_{560} + OH^- \underset{k_a'}{\overset{k_d'}{\rightleftharpoons}} bR_{460} + H_2O$$

The results imply that the Schiff base is titratable in the dark, but its accessibility to external OH^- ions is limited. The limitations in the significance of the "apparent" value of pK_a = 13.3 observed for the Schiff base titration are discussed in light of possible alterations in the structure of bR resulting from the parallel titration of other protein groups. It is suggested that a light-induced pK_a change of at least nine units takes place during the photocycle of light-adapted bR.

Bacteriorhodopsin (bR), the single protein in the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump leading to ATP synthesis [see Stoeckenius et al. (1979) for a comprehensive review]. The chromophore of the light-adapted form of bacteriorhodopsin absorbing at 570 nm (bR_{570}) is *all-trans*-retinal bound to the ϵ-amino group of a lysine via a protonated Schiff base. The dark-adapted modification absorbs at 560 nm (bR_{560}) and contains a 1:1 ratio of 13-*cis*- and *all-trans*-retinal. It appears that the protonated polyene structure not only is responsible for the visible absorption of the pigment but also is directly involved in the proton pump mechanism [see Stoeckenius et al. (1979) and Ottolenghi (1980) for recent reviews].

Various models for proton pumping in bR have been recently discussed (Kalisky et al., 1981). The analysis led to a class of models in which pumping is exclusively based on light-induced pK_a changes in the protein. A specific mechanism was suggested, based on the conclusion that during the photocycle the pK_as of the Schiff base and of a neighboring tyrosine residue are both shifted (from above 12 and $\simeq$10, respectively) to <5.

The rates of deprotonation and reprotonation of the Schiff base and its pK_a are also relevant to understanding the hydrogen–deuterium exchange mechanism in bR (Ehrenberg et al., 1980; Doukas et al., 1981). The problem is closely associated with the accessibility of the Schiff base to protons and to water molecules.

It is thus evident that proton-transfer phenomena directly involving the Schiff base play a primary role in the function of bacteriorhodopsin. However, the irreversible bleaching of the pigment at high pH (above $\simeq$12) has made it impossible to confirm that the Schiff base is titratable in the dark and has also prevented the determination of the pK_a of the retinyl chromophore. In the present work we have carried out a series of continuous and stopped-flow experiments aiming to determine the pK_a value as well as to observe the kinetics of deprotonation and reprotonation of the Schiff base.

Experimental Procedures

All experiments were carried out with 1–10 μM suspensions of dark-adapted bacteriorhodopsin in purple membranes isolated from *Halobacterium halobium* strain M1. The preparation of purple membrane suspensions and the stopped-flow technique (using a Durrum mixing cell and a Beckman spectrometer) have been previously described (Becher & Cassim, 1975; Druckmann et al., 1979). Stopped-flow kinetic traces were obtained with a Biomation 610 transient recorder Absorption spectra were recorded on Cary 14 and Cary 219 spectrophotometers.

The low-temperature resonance Raman apparatus has been discussed elsewhere (Aton et al., 1980). Samples of dark-adapted bR were brought to a pH (or pD in the case of the

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