THE PHYSICAL STATE OF NEWLY SYNTHESIZED RNA*

By James Bonner, Ru-chih C. Huang, and Nirmala Maheshwari

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

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We have earlier reported the enzymatic synthesis of RNA from the four riboside triphosphates by a DNA-containing nucleoprotein of pea embryos.[1] Since the nucleoprotein contains the bulk of the tissue DNA and since it resembles in composition and properties the chromatin obtained directly from the nuclei of pea embryos,[2] we refer to the synthetically active material as chromatin. The RNA newly formed by our system is not liberated as free polynucleotide chains but remains associated with the DNA-containing nucleoprotein from which it may be liberated by heat. The present paper is concerned with the nature of this complex.

Materials and Methods.—Preparation of chromatin: Enzymatically active chromatin (DNA-containing nucleoprotein) was obtained from the embryonic axes of germinating pea seeds. Pea seeds, variety Alaska, were germinated for *ca.* 46 hr, at *ca.* 20°C. The cotyledons were then removed from the growing embryonic axes, which were immediately chilled, sterilized with 100 × diluted Clorox, and ground for 1 min in a Waring Blendor with 1.5 volumes of homogenizing medium (0.25 *M* sucrose, 0.001 *M* $MgCl_2$, and 0.05 *M* tris buffer, pH 8.0). The homogenate was filtered twice through miracloth and the resultant filtrate centrifuged at 4,000 × *g* for 30 min. The chromatin of the pellet was scraped from the underlying hard-packed starch, resuspended in grinding medium, and washed twice by centrifugation at 10,000 × *g* for 10 min. This was followed by two further washings in 0.25 *M* sucrose and one in 0.05 *M* tris buffer, pH 8. The final pellet was suspended in 0.05 *M* tris buffer, pH 8.0, and, frequently, dialyzed against the same buffer for 16 hr. Each kilogram fresh weight of tissue treated in this way yields 400 to 500 milligrams of nucleoprotein, composed of protein, DNA, and RNA, in the ratios of approximately 10:1:0.25.

Determination of RNA, DNA, and protein: Quantitative determination of RNA was generally according to Ts'o and Sato.[3] From the alkaline hydrolysate (0.3 *N* KOH, 18 hr, 37°C), the DNA was precipitated with perchloric acid (0.5 *N*). RNA was determined upon the perchloric-soluble supernatant by optical density and checked by the orcinol method of Dische and Schwartz.[4] DNA was determined by the diphenylamine method of Burton[5] or by optical density of the DNA hydrolysate (0.5 *N* perchloric acid, 90°C, 10 min).

Preparation of nucleoprotein complex containing freshly synthesized C^{14}-labeled RNA: For the preparation of material containing freshly synthesized RNA, 1 ml of enzyme prepared as above and containing approximately 1 mg DNA was incubated for 10 min in an incubation mixture containing tris buffer, pH 8, 200 micromoles; $MgCl_2$, 20 micromoles; cysteine, 20 micromoles; GTP, CTP, and UTP, each 2 micromoles; ATP (8-C^{14}), 3 micromoles (4 microcuries), in a total volume of 2 ml. In each preparation, 20 to 40 incubation mixtures such as that described above were separately incubated and combined at the end of the incubation period. It was found unnecessary to include sodium fluoride in the incubation mixture, since the present preparation has negligible ATPase activity. At the end of the incubation period, the reaction mixture was immediately cooled to 0° and centrifuged for 30 min at 12,000 × *g*, and the pellet was resuspended in standard saline citrate (NaCl 0.15 *M*, Na citrate 0.015 *M*) and dialyzed for 48 hr against continued changes of external solution to remove the bulk of the unincorporated labeled ATP.

Standard assay for amount of label contained in RNA: The amount of C^{14} label contained in RNA was determined by a standard procedure as follows. To the reaction mixture, or aliquot, was added 5 ml cold 5% TCA per ml reaction mixture. The acid-insoluble precipitate was washed once with cold 5% TCA and once with ethanol ether (3:1) and was subsequently extracted twice with 5 ml portions of 10% NaCl, at 100°C, for 30 min. At each extraction, 4 mg of carrier RNA was added. The combined NaCl extracts were then equilibrated with 2 mg of unlabeled ATP for 30 min at room temperature. The RNA was next precipitated by addition of absolute alcohol, final concentration 70%, and the precipitate washed twice with cold 5% TCA. The

precipitate was then redissolved in 1 ml of water with a trace of ammonia. The radioactivity of the RNA was then determined in a gas flow Model D-47 Nulcear Chicago counter on 0.2 or 0.4 ml aliquots.

Nature of labeling in the RNA thus synthesized: It should be noted that the RNA synthesized by our system is internally labeled and does not consist merely of terminal addition of AMP residues to existing polynucleotide chains. That this is so is shown by the data of Table 1, which

TABLE 1

Treatment of RNA	Amount of C^{14}-Labeled AMP Present in Freshly Synthesized RNA ($\mu\mu$m) Initially ($\mu\mu$moles)	After treatment and dialysis ($\mu\mu$moles)
Control	380	342
Periodate	380	317

concerns an experiment in which labeled RNA, prepared as described above, was subjected to treatment for the removal of the terminal nucleoside residue. The labeled RNA was first incubated for 30 min with 1×10^{-3} *M* Na periodate to oxidize to the dialdehyde the ribose of the terminal nucleoside. After completion of oxidation, the mixture was adjusted to pH 10 and incubated at 25° for one hr. The data of Table 1 show that this procedure, which is sufficient for complete removal of the terminal nucleoside, results in insignificant removal of activity from the present labeled RNA. They also demonstrate that the bulk of the labeled AMP incorporated into freshly synthesized RNA by pea embryo chromatin is not terminal, since it is not removed by periodate treatment followed by hydrolysis.

Experimental Results.—That the freshly synthesized and labeled RNA remains closely associated with the DNA-containing nucleoprotein is indicated first of all by the fact that the labeled RNA accompanies the chromatin to the pellet during centrifugation at $10,000 \times g$. This is shown in the data of Table 2. The data of Table 2 further show that the newly formed RNA which accompanies the chromatin

TABLE 2

Fraction	$\mu\mu$moles Labeled RNA* Before RNAase treatment	After RNAase† treatment
Pellet ($10,000 \times g$)	524	513
Supernatant	149	15

* Per sample of chromatin containing 1.7 mg DNA.
† RNAase treatment: 50 μg/ml, 60 min at 37°C.

to the pellet is wholly resistant to attack by RNAase (50 μg per ml, 1 hr at 37°), in a concentration and time and treatment sufficient to degrade to TCA-soluble material 90% or more of free RNA.[1] It is clear therefore that the newly formed RNA which accompanies the chromatin is protected from RNAase in some way and may be spoken of as being bound in complex of some form.

That portion of the newly synthesized RNA which is not readily sedimentable is also apparently bound to chromatin, but to chromatin which has been fragmented to smaller pieces, perhaps by the preparative procedures used. This is shown by the data of Table 3. For this experiment, chromatin containing newly formed RNA was subjected to increasingly strong centrifugation. All of the newly formed RNA is sedimented by centrifugation at 38,000 rpm for 12 hr, and the material thus sedimented possesses the same composition and specific activity as the more readily sedimentable fractions.

Newly synthesized RNA bound in complex in the chromatin nucleoprotein is released from this complex by the action of DNAase, as is shown by the data of

TABLE 3

Fraction	Range of sedimentation constants of fraction	μμmoles newly formed RNA in fraction	Protein:DNA:RNA ratio of fraction
Original chromatin	—	6,940	10:1.2:0.3
Pellet from 10,000 rpm, 60 min SS-1 head	$S < 1000$	4,648	10:1.4:0.5
Pellet from 38,000 rpm, 60 min SW-39 head	$S < 160$	1,364	10:1.2:0.4
Pellet from 38,000 rpm, 6 hr, SW-39 head	$S < 30$	405	— — —
Pellet from 38,000 rpm, 12 hr, SW-39 head	$S < 15$	242	10:1.1:0.3

TABLE 4

Treatment of chromatin	TCA-precipitable newly synthesized RNA* (μμM)
None	372
DNAase†	416
DNAase followed by RNAase‡	88

* Per sample of chromatin containing 0.9 mg DNA.
† DNAase treatment: 50 μg/ml; $MgCl_2$; $3 \times 10^{-2} M$, 2.5 hr at 37°C.
‡ RNAase treatment: 100 μg/ml, 1 hr at 37°C.

Table 4. By release from complex is meant in this case release of RNA to from susceptible to RNAase. The data of Table 4 show that once the DNA of chromatin has been largely degraded by the action of DNAase, the newly synthesized RNA becomes available for attack by RNAase. It may be concluded therefore that the original complex in which the newly synthesized RNA is present and by means of which it is protected from attack from RNAase involves the DNA of the chromatin. It would appear therefore that the complex in which newly formed RNA is associated with DNA is not in the present instance a complex which is not susceptible to DNAase.[6]

It is known that the association between nucleic acid chains to form the double helix of DNA, or between polyribonucleic acid and polydeoxyribonucleic acid chains to form RNA-DNA hybrid double helices, may be broken by heat and that the stability of the particular secondary structure involved may be characterized by its melting profile in which optical density[7] or optical rotation[8] is plotted as a function of temperature. Such a melting profile, using optical density as the property under observation, is presented in Figure 1 for DNA prepared from pea embryo chromatin by the Sevag process according to Marmur.[9] Since the melting profile is dependent upon the ionic strength in which the melting is conducted,[10] data are given in Figure 1 for two ionic strengths, that of standard saline citrate (0.16 M salt) and that of dilute saline citrate (0.016 M salt). In both cases, the optical density of the preparation increases sharply over a range of about 20°C as the result of dissociation of the DNA. The T_M, or temperature at which half-melting is achieved, is approximately 89°C. at the higher ionic strength, and approximately 71° at the lower ionic strength, in general agreement with melting temperatures found for DNAs of other origin.

The melting profile for the liberation of RNA from the complex in which it is bound in chromatin may now be compared with that of the DNA itself. The melting profile of the newly formed RNA complex was determined by making use of the properties of resistance to RNAase of the RNA bound in complex and susceptibility of free RNA to the same enzyme. For these experiments, aliquots of chroma-

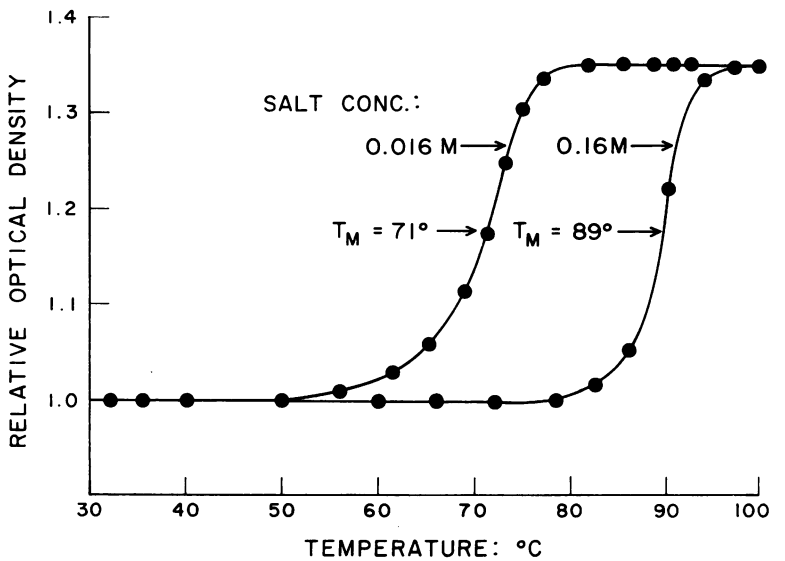

FIG. 1.—Melting profiles as followed by optical density of purified pea embryo DNA in solutions of two different ionic strengths, that of standard saline citrate (0.16 *M*) and that of 10× diluted standard saline citrate (0.016 *M*).

tin containing freshly synthesized labeled RNA were heated for 5 min at each of a range of temperatures. Each aliquot, after it had received its 5-min heat treatment, was inmediately cooled to 0° and subsequently incubated for one hr at 37° with RNAase, and the sample was then analyzed for total TCA-insoluble labeled RNA by the methods given above. The data of Figure 2 show that RNA is liberated to RNAase-susceptible form by treatment of the chromatin at temperatures above about 45°C. At lower temperatures, the newly formed RNA remains resistant to RNAase attack. At temperatures higher than 60°, the RNA becomes essentially completely susceptible to RNAase attack. The T_M or temperature of half-liberation of RNA, by the present criterion, is approximately 50°C. The data of Figure 2 show further, however, that the liberation of freshly formed RNA from its chromatin complex is not affected by alteration of ionic strength of the melting medium. It may be concluded therefore that the original complex is not one involving purely base-pair hydrogen bonds, as in the double-stranded complex of DNA itself. The data of Figure 2 would seem more probably to represent the effect of temperature on denaturation of a protein or proteins of the nucleoprotein complex.

The chromatin nucleoprotein prepared as described above contains endogenous RNA. A portion of this endogenous RNA may be bound to the chromatin in (not formed during incubation *in vitro* with the four riboside triphosphates) trivial manner, but a portion is bound in a manner apparently similar to that in which the newly formed RNA is also bound. Thus the endogenous, nonlabeled RNA of chromatin is resistant to the action of RNAase, as is shown by the data of Table 5, and becomes susceptible to this enzyme as the result of heating of chromatin to 60°C for 5 min.

Treatment of chromatin at 60°C not only releases both endogenous and newly

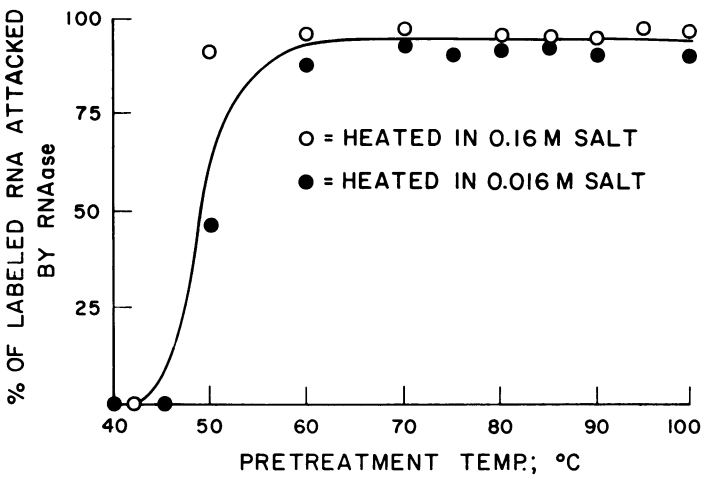

FIG. 2.—Temperature profiles for the release to RNAase-sensitive form of C^{14}-labeled newly synthesized RNA from the RNAase-resistant form in which it is bound in chromatin. For each point, chromatin samples containing approximately 420 $\mu\mu$moles C^{14} AMP in newly synthesized RNA were held for 5 min at the temperature indicated and treated with RNAase (50 μg/ml) for 1 hr at 37°C., and the residual TCA-precipitable RNA was determined.

TABLE 5

Treatment of chromatin	Initial amount of RNA in chromatin (mg)	Amount of RNA degraded by RNAase (mg)	% of RNA released
Chromatin at 0° then RNAase, 37 C, 1 hr	0.14	0.012	8.5
Chromatin at 60°C, 5 min, then RNAase, 37°C, 1 hr	0.14	0.104	74

TABLE 6

Treatment	Material	Amount of Material (mg): Sedimentable at 10,000 × g (mg)	Amount of Material (mg): Not sedimentable at 10,000 × g (mg)
Chromatin held at 0°C	DNA	1.09	0.01
	RNA	0.46	0.10
	protein	12.8	0.37
Chromatin held at 60°C, 5 min	DNA	0.70	0.47
	RNA	0.36	0.24
	protein	13.7	1.3

formed RNA in the sense of converting these materials to RNAase-susceptible form but in addition causes physical release of RNA in the sense that the released RNA is no longer sedimentable with the chromatin by centrifugation at 10,000 $\times$ g. DNA is also liberated from the chromatin by this procedure. As is shown by the data of Table 6, the ratio of RNA to DNA in the liberated and nonreadily sedimentable material is approximately 1 RNA to 2 DNA. This is true whether the particular chromatin preparation used contains much RNA, as in the experiment of Table 6, or relatively less, as in the experiment of Table 3. The thus liberated DNA melts sharply with a T_M in dilute saline citrate (0.016 M salt) of 71°. This DNA is therefore similar to the deproteinized and purified DNA of Figure 1 and is apparently present in double helical conformation. That the RNA

is not bound in any intimate complex whth the DNA of its fraction is indicated by the fact shown in Figure 2 that all of the newly formed RNA of chromatin which has been heated to 60°C is completely degradable by RNAase. It may be concluded therefore that the material released to the supernatant as a result of 60° treatment consists in all probability of a mixture of double helical DNA and free RNA.

Discussion.—It has been shown that the RNA freshly synthesized by the chromatin nucleoprotein complex of pea embryos is not liberated as free RNA, but differs from free RNA in that it is (*a*) sedimented with the chromatin at relatively low centrifugal forces and (*b*) protected by something from susceptibility to attack by RNAase. The freshly synthesized RNA may therefore be said to be present in the chromatin nucleoprotein in the form of a complex. This complex involves the DNA of the chromatin, since degradation of the DNA by DNAase liberates the freshly synthesized RNA. The complex also involves the protein of the chromatin, since deproteinization of the nucleic acid by phenol or by repeated Sevag treatment renders the bulk of the newly formed RNA susceptible to RNAase. RNA is also liberated from the chromatin complex by brief heat treatment at 60°, a temperature lower than the melting temperature of the purified DNA of the material and not influenced, as is the latter, by ionic strength.

It appears, therefore, that both the protein of the nucleoprotein and its DNA are involved in the complex in which the newly formed RNA is synthesized and held. Further hints concerning the nature of this complex are provided by the ratio in which RNA and DNA are liberated from the chromatin by treatment at 60°. This ratio is sufficiently close to 1:2 to invite suspicion. The RNA and DNA thus liberated are no longer in intimate association but behave as free RNA and DNA. The RNA, both newly formed and endogenous, has, by the treatment, been liberated from the original complex. It appears, therefore, that when this complex is disrupted, RNA and DNA are liberated in a ratio of 1:2. The total complex, in its native form as present in the chromatin, may therefore be concluded to be one in which freshly synthesized RNA and DNA are present in this ratio together with an unspecified and as yet unknown amount of protein, the latter conferring upon the complex as a whole a portion of its properties.

Summary.—The RNA enzymatically synthesized from the four riboside triphosphates by isolated chromatin of pea embryos remains bound to the chromatin in the form of an RNA-DNA-protein complex. RNA thus bound is resistant to attack by RNAase but may be released to an RNAase-susceptible form either by (*a*) degradation of the DNA of the complex by DNAase or (*b*) heating of the complex to 60°C for a brief period. Since the temperature which liberates the newly formed RNA is lower than that required to melt the DNA of the chromatin and, unlike the latter, is little influenced by ionic strength, it is concluded that the release of newly formed RNA by heat is attributable to protein denaturation.

DNA as well as RNA is released from chromatin by brief treatment at 60°C. From the stoichiometry of their appearance, it is concluded that the original complex consists of RNA and DNA in a ratio of 1:2 together with protein.

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THE CHEMICAL STRUCTURE AND ENZYMATIC FUNCTIONS OF BOVINE PROCARBOXYPEPTIDASE A

BY JAMES R. BROWN, DAVID J. COX, RODERICK N. GREENSHIELDS, KENNETH A. WALSH, MAKOTO YAMASAKI, AND HANS NEURATH

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON

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Procarboxypeptidase A is the zymogen of an exopeptidase, carboxypeptidase A, which liberates from peptides and proteins carboxyl-terminal amino acids whose side chains conform to the specificity requirements of this enzyme.[1] The active enzyme can be isolated from activated bovine pancreatic juice or from drippings of fresh pancreas glands by a procedure originally described by Anson;[2] alternatively, the enzyme can be isolated from acetone powder of unactivated beef pancreas glands by the procedure of Allan,[3] which is now undergoing further modifications and refinements. Carboxypeptidase A is a metalloenzyme which is inhibited by 1,10 phenanthroline and other metal chelating agents, zinc being the metal which is functionally and structurally intrinsic to the native enzymes.[4, 5] Several molecular parameters pertaining to size, charge properties, and amino acid composition of the enzyme are well established,[1] but the detailed chemical structure of this enzyme remains to be elucidated. Experiments to this end are part of a group program now in progress in this laboratory and in the laboratories of B. L. Vallee, Harvard Medical School.

Procarboxypeptidase A, the inactive precursor of this enzyme, can be found as such in bovine pancreatic juice, in zymogen granules,[6] and in other subcellular components of the acinar cells of the bovine pancreas, and the metabolic origin and history of this and other pancreatic zymogens are now under investigation in this laboratory by Keller and Cohen.[6] While the presence of procarboxypeptidase A was reported in 1937 by Anson,[2] it was only some 20 years later that its isolation in seemingly pure form was achieved by Keller *et al.*[7] When prepared from an aqueous extract of a pancreatic acetone powder, procarboxypeptidase A has all the properties of a single homogeneous protein, as judged by chromatography on DEAE-cellulose, by sedimentation in the ultracentrifuge, and by free-boundary electrophoresis.

The activation of the zymogen is catalyzed by trypsin and, when properly con-