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Reversible Modification of *Escherichia coli* Ribosomes with 2,3-Dimethylmaleic Anhydride. A New Method to Obtain Protein-Deficient Ribosomal Particles†

José Antonio Pintor-Toro, David Vázquez, and Enrique Palacián*

ABSTRACT: Treatment of *Escherichia coli* ribosomes with the protein reagent 2,3-dimethylmaleic anhydride is accompanied by inactivation of polypeptide polymerization and by dissociation of ribosomal proteins. Regeneration of the modified amino groups at pH 6.0 is followed by reactivation and reconstitution of the ribosomes. Prior to regeneration of the amino groups, ribosomal particles and split proteins can be separated by centrifugation, which allows the preparation of new protein-deficient particles. The ribosomal particles obtained by three successive treatments with 2,3-dimethylmaleic anhydride at a molar ratio of reagent to ribosome equal to 16 000 lack proteins S1, S2, S3, S5, S10, S13, S14, L7, L8, L10, L11, L12, and L20 and have lost part of proteins S4, L1, L6, L16, and L25. This new procedure to obtain protein-deficient ribosomal particles is mild and might be useful to dissociate other protein-containing structures in addition to ribosomes.

The protein reagents succinic anhydride, maleic anhydride, and DMMA[1] modify amino groups substituting a negative charge for a positive one, which brings about a drastic change in electrostatic properties frequently accompanied by dissociation of proteins into their subunits (Means & Feeney, 1971; Klotz & Keresztes-Nagy, 1962; Freisheim et al., 1967). Amino groups modified by DMMA can be easily regenerated at moderate low values of pH (Dixon & Perham, 1968).

Modification of *Escherichia coli* ribosomes by succinic, maleic, and acetic anhydrides is followed by inactivation of polypeptide polymerization, peptidyl transferase, and elongation factor G-dependent GTPase, dissociation of the 70S ribosomes into 50S and 30S subunits, and, probably, separation of individual proteins (Pintor-Toro et al., 1978). Since DMMA can be easily removed with regeneration of the modified amino groups, this reagent might be used to dissociate proteins from the ribosome and to obtain the corresponding protein-deficient particles.

The present paper reports a new method to prepare ribosomal "cores" and split proteins based on the reversible modification of protein amino groups with the reagent DMMA, which causes dissociation of ribosomal proteins in a fairly specific way.

Materials and Methods

Preparation of Ribosomes and Assay of Activities. Ribosomes were prepared from *E. coli* MRE 600 by grinding the cells with alumina, followed by differential centrifugation. Ribosomes were washed three times with 1 M NH_4Cl, suspended in 5 mM Tris-HCl (pH 7.8), 20 mM magnesium acetate, 500 mM NH_4Cl, 2 mM dithiothreitol, 0.5 mM EDTA and 50% (v/v) glycerol, at a ribosomal concentration of 75 mg/mL, and kept at −20 °C (Modolell & Vázquez, 1973). The 30S subunits were prepared by zonal centrifugation (Eikenberry et al., 1970). Poly(U)-directed polyphenylalanine synthesis was determined in a crude system containing S-100 extract (Nirenberg & Matthaei, 1961). Peptidyl transferase was estimated by the "fragment reaction" assay, using C-(U)-A-C-C-A-(Ac[^{3}H]Leu) and puromycin as substrates (Monro, 1971).

Modification of Ribosomes, Separation of Ribosomal Particles from Split Proteins, and Regeneration of the Modified Groups. Prior to treatment with DMMA, 0.2 mL of stored ribosomes (15 mg) was diluted with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K^+) (Hepes) (pH 8.2), 20 mM magnesium acetate, 0.5 mM dithiothreitol, and 0.5 mM EDTA, to a final volume of 2 mL. To this solution, the reagent (300 mg/mL of DMMA in dioxane) was added stepwise in aliquots of 5 μL, and the pH was maintained at 8.2 by addition of 0.5 M KOH. The treatment took place at room temperature and was complete in 1 h. To separate the ribosomal particles from the split proteins, immediately after DMMA treatment the volume of the preparation was increased to 5 mL with 50 mM Tris-HCl (pH 8.2), 20 mM magnesium acetate, 50 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM EDTA and centrifuged for 4.5 h at 2 °C and 64 000 rpm in a Beckman SW65 rotor. After centrifugation, the supernatant was collected and the sediment suspended in 2 mL of the above mentioned solution. To regenerate the modified amino groups, the corresponding preparation was dialyzed for 48 h at 0–5 °C against 20 mM

† From the Instituto de Bioquimica de Macromoléculas, Centro de Biología Molecular, Consejo Superior de Investigaciones Cientificas, Universidad Autónoma de Madrid, Canto Blanco, Madrid-34, Spain. *Received January 3, 1979.* This work was supported by institutional grants from Comisión Administradora del Descuento Complementario (Instituto Nacional de Previsión) and by personal grants from Lilly Indiana of Spain and Essex Laboratories.

[1] Abbreviation used: DMMA, 2,3-dimethylmaleic anhydride.

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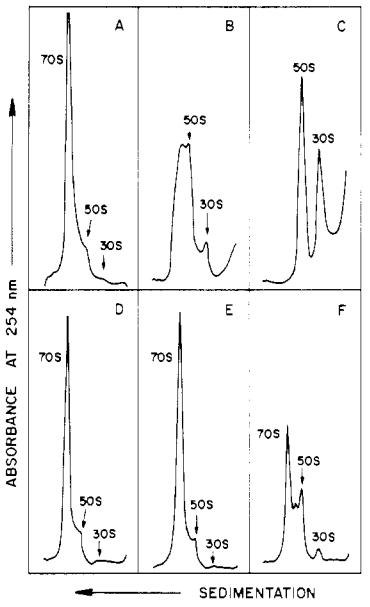

FIGURE 1: Sedimentation patterns of DMMA-treated ribosomes undialyzed (A, B, and C) and dialyzed at pH 6.0 (D, E, and F). Ribosomes were treated without (A and D) and with DMMA at molar ratios of reagent to ribosomes equal to 2100 (B and E) and 10 500 (C and F). Immediately after DMMA treatment, preparations D, E, and F were dialyzed at pH 6.0.

sodium cacodylate (adjusted to pH 6.0 with HCl), 20 mM magnesium acetate, 60 mM NH_4Cl and 0.5 mM EDTA.

Sedimentation Analysis. Sedimentation was studied by centrifugation in linear 5–20% sucrose gradients containing 10 mM Tris-HCl (pH 7.8), 10 mM magnesium acetate, and 100 mM NH_4Cl. The ribosomal preparations (40 pmol) were centrifuged in a Sorvall AH 650 rotor at 48 000 rpm and 4 °C for 1.25 h. The distribution of particles along the gradient was determined with an Isco density fractionator.

Electrophoresis and Quantitative Determination of Split and "Core" Proteins. Proteins from ribosomal particles were extracted with 67% (v/v) acetic acid, at a final magnesium concentration of 0.2 M (Barritault et al., 1976). After 45 min of treatment with acetic acid at 0 °C, rRNA was removed by centrifugation. The supernatant containing the ribosomal proteins was dialyzed for 12 h at 0–5 °C against 0.5% acetic acid and lyophilized. After dialysis at pH 6.0, the split proteins fractions were also dialyzed against 0.5% acetic acid and lyophilized prior to protein determination or gel electrophoresis. Protein concentration was determined as described by Lowry et al. (1951). Two-dimensional polyacrylamide gel electrophoresis was performed according to Howard & Traut (1974).

Results

Modification of *E. coli* ribosomes with DMMA is accompanied by inactivation and changes in sedimentation that are reversed by regeneration of the modified amino groups at pH 6.0. Figure 1 shows the sedimentation patterns of two DMMA-treated ribosomal preparations (B and C) and the untreated control (A). Modification was accompanied by dissociation of the 70S ribosomes into 50S and 30S subunits, in agreement with previous results obtained with other acid anhydrides (Pintor-Toro et al., 1978). The treated preparations had less than 5% of the polypeptide polymerization activity of the untreated control. After dialysis at pH 6.0, the DMMA-treated preparations showed increase in 70S ribosomes and decrease in 50S and 30S subunits (Figure 1, E and F). In the preparation treated with the highest concentration of reagent (F), the recovery of the original sedimentation

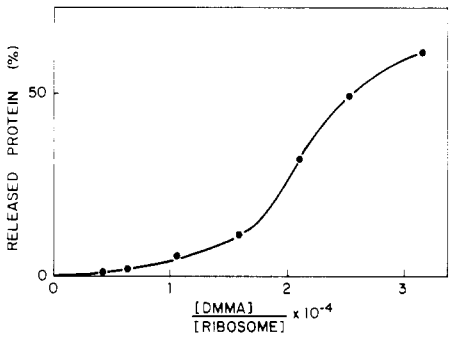

FIGURE 2: Protein released upon DMMA treatment. Ribosomes were treated at the indicated molar ratios of reagent to ribosomes. After separation from the ribosomal particles by centrifugation, the released proteins were dialyzed at pH 6.0 and lyophilized, and their amount was determined, as indicated under Materials and Methods. Protein released is expressed as percentage of the total protein content of the corresponding ribosomal preparation not treated with DMMA. The protein content of the ribosomal particles separated from each DMMA-treated preparation was also determined. Its value plus that of the corresponding released proteins agrees within 5% with that of the untreated control.

pattern was partial, and a peak intermediate between 70S ribosomes and 50S subunits was observed. This intermediate peak might result from a rapid association–dissociation equilibrium between 50S and 30S subunits with decreased affinity for each other. After dialysis at pH 6.0, polypeptide polymerization activity was also recovered (90% of the control in preparation E and 60% in the preparation F, Figure 1). Incubation of ribosomes with dimethylmaleic acid at a molar ratio of this compound to ribosome equal to 20 000, under the same conditions used with the anhydride, did not have any observable effect on polypeptide polymerization, indicating that the effects of DMMA treatment are not produced by the hydrolysis product of DMMA.

Treatment of ribosomes with DMMA is followed by dissociation of proteins that can be separated from the corresponding particles by centrifugation. Figure 2 shows the percentage of total ribosomal proteins released after treatment with DMMA at different molar ratios of reagent to ribosomes. At molar ratios higher than 25 000, more than 50% of the proteins were dissociated from the ribosome.

The ribosomal particles separated from the released proteins by centrifugation, and dialyzed at pH 6.0 to regenerate the modified amino groups, showed decreased activity as compared with the untreated control. The polypeptide polymerization and peptidyl transferase activities of ribosomal particles obtained from preparations treated with different molar ratios of reagent to ribosomes are shown in Figure 3. At low molar ratios, little effect was observed on polypeptide polymerization, while an increase in activity with respect to the control was found for peptidyl transferase. At higher concentrations of reagent, both activities disappeared in a parallel way, and at a molar ratio of reagent to ribosome equal to 31 500, the ribosomal particles were practically devoid of activity. When the corresponding released proteins were added to the ribosomal particles, under reconstitution conditions, partial recovery of polypeptide polymerization activity was observed in all preparations (Figure 3).

The ribosomal particles isolated by centrifugation after DMMA treatment and dialyzed at pH 6.0, in order to regenerate the modified amino groups, present a sedimentation behavior different from that of the untreated control. Figure 4 shows that the lack of the proteins released by DMMA treatments produced dissociation of 70S ribosomes into subunits with sedimentation coefficients about 50 S and 30

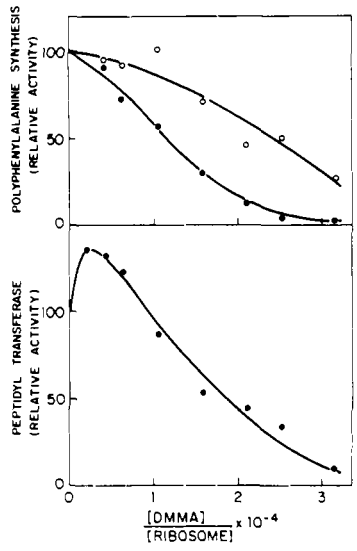

FIGURE 3: Polyphenylalanine synthesis and peptidyl transferase activity of ribosomal particles obtained after DMMA treatment. Ribosomes were treated with DMMA at the indicated molar ratios of reagent to ribosomes, the ribosomal particles were separated from the released proteins by centrifugation, and each fraction was dialyzed at pH 6.0. Immediately before polyphenylalanine synthesis assay, aliquots of the ribosomal particles with (○) and without (●) eight times the corresponding amount of homologous split proteins were incubated at 37 °C for 15 min with all the components of the reaction mixture for polyphenylalanine synthesis except S-100. Peptidyl transferase activity was assayed in aliquots of the ribosomal particles with neither preincubation at 37 °C nor addition of released proteins. The controls (100% activity) were subjected to the same treatments as the corresponding samples but in the absence of DMMA. The activities of the controls were 23 molecules of [^{3}H]phenylalanine incorporated and 4.5×10^{-3} molecules of acetyl[^{3}H]leucylpuromycin synthesized per ribosome.

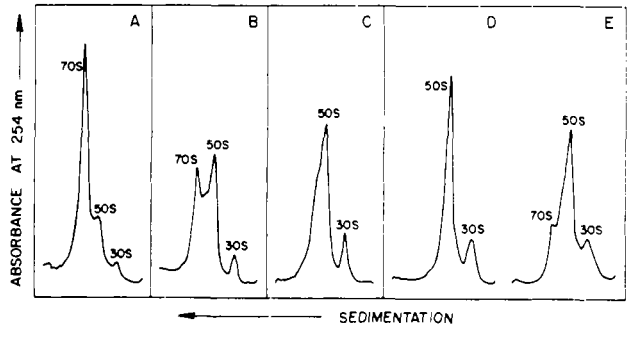

FIGURE 4: Sedimentation patterns of ribosomal particles obtained after DMMA treatment. Ribosomes were treated without (A) and with DMMA at the following molar ratios of reagent to ribosomes: 10500 (B), 15750 (C), and 25200 (D and E). Immediately after DMMA treatment, the ribosomal particles were separated from the released proteins by centrifugation and dialyzed at pH 6.0. Prior to sucrose gradient centrifugation, the ribosomal particles treated with the highest reagent concentration were incubated at 37 °C for 10 min with the corresponding amount of the homologous split proteins, after regeneration of their amino groups by dialysis at pH 6.0 (E).

S. When the ribosomal particles were incubated with the released proteins prior to centrifugation, there was partial reconstitution of 70S ribosomes, even in the preparation treated with the highest concentration of reagent (molar ratio of DMMA to ribosomes equal to 25000).

To study the specificity of protein dissociation, electrophoresis of the released proteins and of the proteins present in the corresponding ribosomal particles was performed in preparations treated with different molar ratios of DMMA to ribosomes. At a molar ratio equal to 16000 (Figure 5C), the fraction of released proteins showed proteins S2, S3, S5, L1, L3, L6, L7, L8, L9, L11, and L12, as well as traces of other proteins. At molar ratios of reagent to ribosomes lower than 16000, the same proteins were released although in lower amounts (Figure 5B), while at higher molar ratios many additional proteins were simultaneously released (Figure 5, D and E). Electrophoresis of the proteins from the homologous ribosomal particles obtained at a molar ratio of DMMA to ribosomes equal to 16000 showed that, although the intensity of the spots corresponding to the released proteins had notably decreased, they had not been completely removed. To achieve a more complete removal, reagent treatment, separation of ribosomal particles and regeneration of the amino groups were repeated three times at the molar ratio of reagent to ribosome used before, each time the ribosomal particles obtained in one step being subjected to the next. Figure 5F shows the electrophoresis of the proteins retained in the ribosomal particles after the third extraction. It can be seen that proteins S1, S2, S3, S5, S10, S13, S14, L7, L8, L10, L11, L12, and L20 have been completely removed from the particles, and proteins S4, L1, L6, L16, and L25 have been lost in a large proportion.

Table I: Reconstitution of Ribosomes from Ribosomal "Cores" Obtained by Three Successive Treatments with DMMA[a]

preparation	additions	poly(Phe) synth (pmol of Phe incorp/ ribosome in 30 min)
control		0.86
control	30 S	8.0
"core"		0.58
"core"	split proteins	0.57
"core"	30 S	1.7
"core"	split proteins, 30 S	4.1

[a] Reconstitution was assayed as recovery of polyphenylalanine synthesis. The ribosomal "cores" were obtained after three successive cycles of modification at a molar ratio of DMMA to ribosomes equal to 16000 (see legend to Figure 5). The control was prepared by subjecting the ribosomes to exactly the same treatments as the "cores" but in the absence of DMMA. The preparations (1 μM "cores" or control ribosomes) were incubated for 90 min at 50 °C in the presence or absence of the total split proteins obtained in the preparation of the "cores" (the amount of split proteins added was three times that dissociated from the "cores" to which they were added), in 50 mM Tris-HCl (pH 7.8), 20 mM magnesium acetate, 400 mM NH_4Cl, and 2 mM dithiothreitol. Immediately before assay of polyphenylalanine synthesis, aliquots of the preparations were incubated with or without native 30S subunits (molar ratio of 30S subunits to "cores" or control ribosomes equal to 2), at 37 °C for 15 min with all the components of the reaction mixture for polyphenylalanine synthesis except S-100.

The control ribosomes corresponding to the ribosomal particles obtained by three successive treatments with DMMA lose most of the activity of polyphenylalanine synthesis during preparation of the particles (Table I). However, incubation with 30S subunits produces recovery of activity, indicating that the 30S subunit of the control ribosomes has been inactivated by the long manipulations to which they and the DMMA-treated sample are subjected during preparation of the ribosomal "cores", independently from the inactivation produced by the DMMA treatments. The ribosomal "cores" have little activity and this activity is not increased after incubation with the corresponding split proteins. When the ribosomal "cores" are supplemented with native 30S subunits, the activity is enhanced and prior incubation with the split proteins is accompanied by an additional increase in activity to about 50% of the activity of the 30S subunit-supplemented control. In summary, the preparation of the ribosomal particles is accompanied by inactivation of the 30S subunits independent of DMMA treatment, while separation of split proteins from

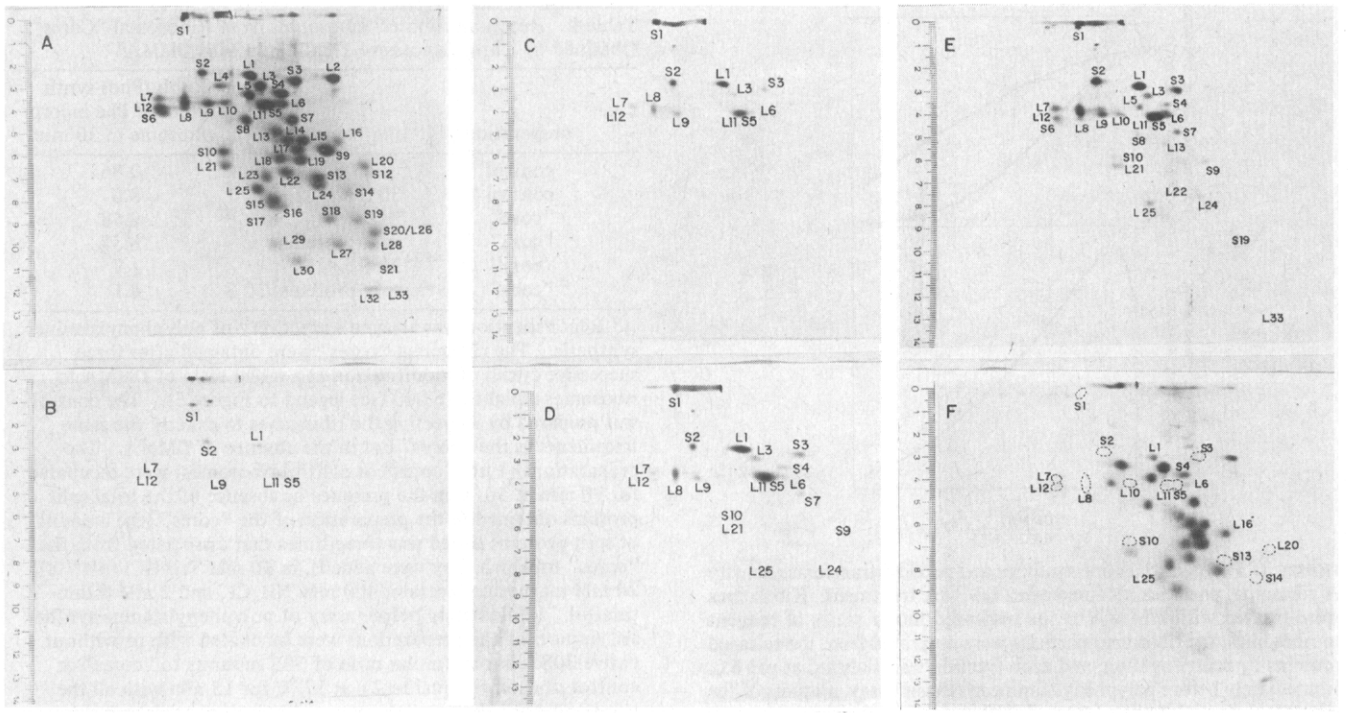

FIGURE 5: Electrophoresis patterns of the proteins released and those bound to ribosomal particles after DMMA treatments. (A) Proteins of untreated control. Split proteins obtained by DMMA treatment of the ribosomes at a molar ratio of reagent to ribosome equal to 6400 (B), 16000 (C), 21300 (D), and 25600 (E), followed by separation from the corresponding ribosomal particles by centrifugation and regeneration of the amino groups at pH 6.0. (F) Proteins from the ribosomal particles obtained after three successive cycles of modification. Each cycle consisted of DMMA treatment at a molar ratio equal to 16000, separation of ribosomal particles, and regeneration of the amino groups. The ribosomal particles obtained in one cycle were subjected to the next.

the 50S subunits produces inactivation of the particles, this inactivation being partially reversed by addition of the split proteins.

Discussion

Treatment of ribosomes with DMMA in the range of concentrations used in this work should be accompanied by modification of a large number of lysine residues. Although no determination of the number of amino groups modified by DMMA has been done, because of the lability of the product and the necessity of prior separation and unfolding of the ribosomal proteins, the nonremovable similar reagent succinic anhydride modifies a large proportion of the protein amino groups under conditions similar to those used with DMMA (about 40% of the total protein amino groups react with succinic anhydride at a molar ratio of reagent to ribosome equal to 20000) (Pintor-Toro et al., 1978). The chemical modification of the amino groups of ribosomal proteins with DMMA seems to be completely reversible, because of the known chemical behavior of the reagent (Dixon & Perham, 1968), the reversibility of inactivation and of the changes in sedimentation, and the neat electrophoresis patterns of the ribosomal proteins from treated preparations. These patterns do not show additional spots, while the change in charge brought about by the modification should be accompanied by a change in electrophoretic mobility, as it was found with succinic anhydride (Pintor-Toro et al., 1978). It is remarkable the high reversibility of the modification obtained with DMMA as well as that of the inactivation, considering that the molar ratios of reagent to ribosomes used in this work are about 100 times higher than those needed to inactivate 30S subunits with maleic anhydride (Cantrell & Craven, 1977) and 70S ribosomes with succinic anhydride (Pintor-Toro et al., 1978).

The results presented in this paper show that the reversible modification of the amino groups in ribosomes with DMMA is a useful method to specifically dissociate ribosomal proteins. This method could also be applied to dissociate other protein-containing particles. The procedure is mild, and the separated proteins probably retain a high degree of secondary and tertiary structure.

Since the dissociation of proteins is achieved by means different from those used so far in the preparation of ribosomal "cores" (Maglott & Staehelin, 1971; Spitnik-Elson & Greenman, 1971; Homann & Nierhaus, 1971; Hamel et al., 1972), it was reasonable to expect the production of new ribosomal particles with different protein composition and properties. In fact, the ribosomal "cores" obtained by three successive treatments with DMMA present a protein content different from those already described (Nierhaus & Montejo, 1973; Sander et al., 1975; Bernabeu et al., 1976). The ribosomal "cores" obtained with DMMA are relatively homogeneous as shown by electrophoresis of the unreleased proteins, and the study of their properties is interesting in itself. Good reconstitution by addition of the released proteins would increase their value in the study of the structure–function relationships in the ribosome. Reconstitution of the ribosomes from the ribosomal "cores" obtained with DMMA, assayed as recovery of polyphenylalanine synthesis, indicates that the 30S subunit is inactivated because of the long operations to which the ribosomes are subjected (about 9 days) to prepare the "cores" and is independent of the DMMA treatments. With respect to the 50S subunit, this can be reconstituted from the "cores" and the split proteins giving 50% of the activity of the control. In conclusion, the ribosomal particles obtained by three successive cycles of treatment with DMMA are fairly homogeneous and can reconstitute ribosomes with a reasonable yield. These ribosomal "cores" and new ones obtained with DMMA, the study of which has already been undertaken in our laboratory, might be useful to establish relationships

between structure and function in the ribosome.

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Chromatin Structure of the 5S Ribonucleic Acid Genes of *Xenopus laevis*†

Stephen E. Humphries,‡ Dallan Young, and Dana Carroll*

ABSTRACT: Staphylococcal nuclease digestion has been used to investigate the structure of bulk chromatin and of that containing the genes for 5S RNA in two tissues from *Xenopus laevis*. In red blood cells, the nucleosome repeat length of the majority of the chromatin is 189 ± 4 bp. In nuclei prepared from liver cells, two populations of nucleosome repeat lengths were detected. The majority of the chromatin has a repeat length of 178 ± 5 bp, but, at late times of digestion with staphylococcal nuclease, a fraction of chromatin with a repeat length of 155 ± 8 bp is apparent. Oocyte-type 5S DNA comprises 0.38% of the *X. laevis* genome, or about 30 000 repeating units per diploid cell, but is not transcribed in somatic tissues. During nuclease digestion, the fate of these sequences has been monitored by hybridization with specific probes. By using the blotting technique of E. M. Southern ((1975) *J. Mol. Biol. 98*, 503–517), the nucleosome repeat length of 5S DNA is 175 ± 5 bp in both blood and liver, suggesting that these specific genes may have a constant chromatin structure, independent of the organization of bulk chromatin in the two tissues. In addition, 5S DNA is less susceptible to staphylococcal nuclease than is bulk DNA in nuclei from both cell types. Quantitative hybridization techniques demonstrate that, with increasing degrees of digestion, high molecular weight nucleosome multimers are enriched up to threefold in 5S DNA sequences. Concomitantly, the 5S DNA content of monomer DNA from these digests is reduced. Calculations indicate that the oocyte 5S DNA sequences, which in the tissues studied are transcriptionally silent, are cut by staphylococcal nuclease at 60–80% of the rate that the bulk DNA is cut.

It is now clear that the nucleosome structures first described in rat liver (Hewish & Burgoyne, 1973; Noll, 1974) and in chicken erythrocytes (Olins & Olins, 1974) can be found as the basic unit of chromatin structure in all eukaryotes (for review, see Kornberg, 1977). Current models envision a repeating array of structures, consisting of 2 molecules each of the histones H2A, H2B, H3, and H4, and with approximately 140 bp of DNA wrapped round the protein core (Sollner-Webb & Felsenfeld, 1975; Axel, 1975; Ramsay-Shaw et al., 1976). This core structure seems to be invariant in size and histone content in eukaryotes (Noll, 1976; Compton et al., 1976; Morris, 1976; Lohr et al., 1977). However, the spacing of the nucleosomes in the chromatin varies considerably in different organisms, from 160 bp in fungi (Noll, 1976; Morris, 1976) to 241 bp for sea urchin sperm (Spadafora et al., 1976), due to different lengths of internucleosome DNA. This DNA, which is associated with the histone H1 (Noll & Kornberg, 1977), is the site of attack by nucleases (Noll, 1974) which cleave to give the characteristic banding pattern of multiples of the monosome DNA size.

It has been suggested that longer internucleosome distances are correlated with transcriptionally inactive chromatin (Morris, 1976; Thomas & Thompson, 1977), perhaps due to differences in the histone primary sequence (Morris, 1976) or to chemical modification of the protein (Thomas & Thompson, 1977; Allfrey, 1971). It is, however, clear that both transcribing and nontranscribing DNA sequences can be found

† From the Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center, Salt Lake City, Utah 84132. *Received January 23, 1979.* S.E.H. was supported by a fellowship from the Anna Fuller Fund, and D.C. and D.Y. were supported by grants from the National Institutes of General Medical Sciences.

‡ Present address: Department of Biochemistry, St. Mary's Hospital Medical School, University of London, London W.2, England.

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