METHODOLOGY FOR THE STUDY OF THE TEMPLATE ACTIVITY OF CHROMOSOMAL NUCLEOHISTONE[1]

James Bonner and Ru-chih C. Huang[2]

Division of Biology

California Institute of Technology, Pasadena, California

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For the study of the template activity of chromosomal nucleohistone in DNA-dependent RNA synthesis, it is desirable that the template nucleohistone remain in solution in the RNA synthesis mixture. Two conditions must be met if this is to be achieved. First, the effective concentrations of Mg^{++} and Mn^{++} ions used must not exceed those of the Chamberlin and Berg (1962) RNA synthesis reaction mixture. Secondly, the nucleohistone template must be free of contaminating nonchromosomal proteins. It is often found, however, and occasionally reported (Sonnenberg and Zubay, 1965) that native nucleohistone precipitates from solution in the reaction mixture used for conduct of RNA synthesis. We herewith consider the two conditions described above which must be fulfilled if such precipitation is to be avoided.

The first concerns the composition of the reaction mixture itself. The activity of the enzyme which is commonly used to catalyze DNA-dependent RNA synthesis, RNA polymerase of E. coli, requires

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[2]Present address: Department of Biology, The Johns Hopkins University, Baltimore, Maryland.

the presence of magnesium and/or manganese ions, both of which in appropriate concentrations precipitate nucleohistones from solution. It is of importance, therefore, that their concentration be set at a level which promotes the action of RNA polymerase, but which is still low enough to avoid precipitation of the template. This is true, as we have shown earlier (Huang, Bonner and Murray, 1964) of the RNA synthesis reaction mixture of Chamberlin and Berg (1962), whose composition is shown in Table 1.

Table 1

Compositions of reaction mixtures for the conduct of DNA-dependent RNA synthesis by RNA polymerase of E. coli[1]

Ingredient	Reaction mixture of: Chamberlin and Berg (1962)[2] μmoles/0.25 ml	Sonnenberg and Zubay (1965) μmoles/0.25 ml
Tris, pH 8.0	10	12.5
$MgCl_2$	1	1.25
$MnCl_2$	0.25	0.40
SH-EtOH	3	3.5
ATP, GTP, CTP, UTP	0.1 (each)	0.25 (each)

[1] The reaction mixture used for the conduct of DNA-dependent RNA synthesis catalyzed by chromosomal RNA polymerase is of a composition different from either of those shown below (Huang and Bonner, 1962).

[2] Also used by Huang, Bonner and Murray (1964).

That native nucleohistone remains soluble in this reaction mixture is shown by the data of Table 2. For the experiments of Table 2, thymus nucleohistone was prepared by the methods of Bonner and Huang (1963).

Table 2

Solubility of two types of thymus nucleohistone preparations in RNA synthesis reaction mixtures

System	Visual appearance after addition of nucleohistone to reaction mix.	μg nucleohistone DNA/ml[2] Added	Left in sol. after centrif. for 15 min at 35K, rpm	% of input DNA in supernatant
Nucleohistone prepared from chromatin centrifuged through 1.7 M sucrose				
1. Chamberlin-Berg reaction mixture[1]	clear	108	101	93.5
2. Sonnenberg-Zubay reaction mixture[1]	turbid	108	11.1	10.2
Nucleohistone prepared from chromatin not centrifuged through 1.7 M sucrose				
3. Chamberlin-Berg reaction mixture[1]	turbid	100	3.8	3.8
4. Sonnenberg-Zubay reaction mixture[1]	aggregated	100	4.5	4.5

[1] The β-mercaptoethanol is omitted from the reaction mixture to permit conduct of the Burton diphenylamine assay for DNA.

[2] DNA determined by diphenylamine assay.

This preparation follows the recommendations of Zubay and Doty (1959) with the additional refinement that the crude chromosomal pellet resuspended in 0.01 M tris pH 8 is layered on 1.7 M sucrose and centrifuged through it (22,000 rpm, Spinco #25 rotor, 105 minutes). The resulting gelatinous pellet is resuspended in 0.01 M tris pH 8, dialyzed to remove sucrose, and then sheared in a blendor as described by Zubay and Doty (1959). The sheared chromatin is next centrifuged at 10,000 rpm (Serval SS-34 rotor, 10,000 x g) for 30 minutes. The resulting supernatant, which consists of DNA fully complexed with

histone, constitutes the so-called soluble nucleohistone. Any remaining aggregates are then removed by centrifugation at 35,000 rpm in a Spinco #39 rotor. Of the nucleohistone not pelleted by centrifugation for 30 minutes at 10,000 rpm, 75 to 80% is also not pelleted by centrifugation for 15 minutes at 35,000 rpm.

For the experiment of Table 2, line 1, nucleohistone prepared in the above manner was dissolved at a concentration of 108 μg DNA/ml in reaction mixture of the composition shown in Table 1 which is that of Chamberlin and Berg (1962) (also used by Huang, Bonner and Murray, 1964) but lacking β-mercaptoethanol. The mixture, which remains clear, was then centrifuged at 35,000 rpm in the Spinco #39 rotor for 15 minutes to remove any newly formed aggregates. The top 0.3 ml of the centrifuged solution was next removed and its DNA content determined by the diphenylamine method of Burton (1956) using 0.3 ml of reaction mixture (without nucleohistone) similarly incubated with diphenylamine as blank. Under these circumstances, 93.5% of the input DNA remains in solution in the supernatant, just as previously described (Huang, Bonner and Murray, 1964).

Thymus nucleohistone, even though it remains in solution in the reaction mixture used for RNA synthesis, is essentially inactive as a template. Thus the preparation of Table 2, line 1, was found to be but 0.6% as effective in support of DNA-dependent RNA synthesis with added E. coli RNA polymerase (Huang, Bonner and Murray, 1964) as was deproteinized calf thymus DNA. Increase in the concentrations of magnesium (Mg^{++}) and of manganese (Mn^{++}) above the levels which we have used, and which are those of the Chamberlin-Berg reaction mixture, lead to precipitation of nucleohistone from solution, as has been elegantly demonstrated by Sonnenberg and Zubay (1965). The reaction mixture which these investigators employ (and which they incorrectly describe as identical with those of Huang, Bonner and

Murray (1964) and of Chamberlin and Berg (1962)) possesses the composition shown in Table 1. The data of Table 2, line 2, show that the same nucleohistone preparation which is not aggregated in Chamberlin-Berg reaction mixture becomes turbid in the reaction mixture of Sonnenberg and Zubay (1965), and is 90% removed from solution by 15 minutes of centrifugation at 35,000 rpm in the Spinco #39 rotor.

The nucleoside triphosphates must be present in the reaction mixture of Chamberlin and Berg (also used by Huang, Bonner and Murray) if nucleohistone is to remain soluble in it. Thus, reaction mixtures similar to that of Chamberlin and Berg and of Huang, Bonner and Murray, but lacking the riboside triphosphates (as well as the β-mercaptoethanol) were made up with nucleohistone, prepared as described above, and to a final DNA concentration of approximately 100 μg/ml. Each preparation was then centrifuged for 15 minutes at 35,000 rpm in the Spinco #39 rotor. The top 0.6 ml of each supernatant was then analyzed by optical

Table 3

Insolubility of thymus nucleohistone in reaction mixtures similar to those of Table 1, but lacking nucleoside triphosphates

System	μg nucleohistone DNA/ml[2]		
	Added	Left in solution after centrif. for 15 min at 35K, rpm	% of input DNA in supernatant
1. Chamberlin-Berg reaction mixture[1] plus thymus nucleohistone	108	2.25	2.1
2. Sonnenberg-Zubay reaction mixture[1] plus thymus nucleohistone	108	3.25	3.0

[1] The β-mercaptoethanol (as well as the NTP's) is omitted from the reaction mixture.

[2] DNA determined by absorbancy at 260 mμ.

density at 260 mμ. The data of Table 3 show that both in the Chamberlin-Berg and Huang, Bonner and Murray reaction mixture, and the Sonnenberg-Zubay reaction mixture, nucleohistone is 97 to 98% removed by aggregation in such nucleoside triphosphate-free reaction mixture.

It is clear that the effective concentrations of magnesium and manganese ions in the Chamberlin-Berg, Huang-Bonner RNA synthesis reaction mixture are close to the limit which can be tolerated by thymus nucleohistone without precipitation. In such a reaction mixture a portion of the magnesium and manganese ions are bound by the nucleoside triphosphates. If these are omitted, the nucleohistone precipitates.

The second important condition which must be fulfilled if nucleohistone is to remain soluble in the RNA synthesis reaction mixture is that the nucleohistone must be free of contaminating nonchromosomal protein. It is for this reason that the step in which chromatin is centrifuged through 1.7 M sucrose as described above has been introduced into the procedure for the preparation of soluble nucleohistones. At this step, a major portion of the nonchromosomal protein contained in preparations of crude chromatin is removed (Huang and Bonner, 1962). Crude chromatin, if sheared directly, yields nucleohistone containing contaminating nonchromosomal protein. The presence of such contaminants, which are separable from nucleohistone not only by centrifugation through sucrose but also by zone electrophoresis (Olivera, Huang and Davidson, 1964) leads to aggregation of the nucleohistone under a variety of experimental circumstances. Thus thymus nucleohistone prepared as described above, but without purification by the sucrose centrifugation step, aggregates both in the reaction mixture of Chamberlin and Berg (1962) and in that of Sonnenberg and Zubay (1965) as is shown in lines 3 and 4 of Table 2.

The soluble nucleohistone component of chromatin is, then, soluble

only under a limited spectrum of conditions. It is, however, soluble in RNA synthesis reaction mixture, provided that the nucleohistone is of appropriate purity, and that the reaction mixture contains magnesium and manganese ions in a sufficiently low effective concentration.

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