

Structure of transcriptionally active chromatin

(chromatin fractionation/deoxyribonuclease II/chromatin subunits/ ν -bodies)

JOEL M. GOTTESFELD*, ROBERT F. MURPHY, AND JAMES BONNER

Division of Biology, California Institute of Technology, Pasadena, Calif. 91125

Contributed by James Bonner, September 10, 1975

ABSTRACT Rat-liver chromatin has been fractionated into transcriptionally active and inactive regions [Gottesfeld *et al.* (1974) *Proc. Nat. Acad. Sci. USA* 71, 2193-2197] and the distribution of nuclease-resistant complexes in these fractions has been investigated. About half of the DNA of both fractions is resistant to attack by the endonuclease DNase II. The nuclease-resistant structures of inactive chromatin are DNA-histone complexes (ν -bodies) which sediment at 11-13 S. Template-active chromatin yields two peaks of nuclease-resistant nucleoprotein. These complexes sediment at 14 and 19 S, and contain DNA, RNA, histone, and nonhistone chromosomal proteins. Polyacrylamide gel electrophoresis reveals a complex pattern of chromatin proteins, suggesting that the complexes are heterogeneous in composition.

A regular repeating unit in chromatin was first suggested from the x-ray diffraction studies of Pardon *et al.* (1): a series of reflections were observed in the x-ray patterns of native and reconstituted nucleohistones, but not in the x-ray diffraction patterns of DNA or histones by themselves. It was proposed that the chromatin fiber is organized into a regular supercoil of pitch 100-120 Å. This model, although widely accepted for some time, has now come under question. Olins and Olins (2) have observed regular spacings of chromatin particles (termed ν -bodies) in water-swollen nuclei centrifuged onto electron microscope grids. These particles are 60-80 Å in diameter and are joined by thin filaments 15 Å in diameter. These results have been confirmed and extended by other laboratories (3-5). Nuclease digestion studies also support a subunit or particulate structure for chromatin. Both endogenous (6-7) and exogenous (8-14) nucleases appear to recognize a repeating nucleoprotein unit along the chromatin fiber. Furthermore, chromatin particles isolated from nuclease-treated or sonicated chromatin resemble ν -bodies in the electron microscope (15-17). Thus many lines of evidence support the subunit or "beads-on-a-string" model of chromatin structure (2, 18).

Studies on chromatin structure have been generally carried out with whole chromatin, unfractionated with respect to transcriptional activity. Since only a minor portion of the DNA in any differentiated cell type is ever transcribed into RNA, the properties of unfractionated chromatin reflect primarily the structure of inactive regions. We are interested in whether template-active chromatin is organized as is the inactive region or whether it is in a different conformation. Previous work from this laboratory has shown that it is possible to separate chromatin into transcriptionally active and inactive fractions (19-21). In this communication we report that both fractions contain nuclease-resistant nucleoprotein complexes. However, the nuclease-resistant structures of inactive chromatin are DNA-histone complexes, while the nu-

clease-resistant structures of active chromatin are complexes of DNA, RNA, histone, and nonhistone chromosomal protein.

MATERIALS AND METHODS

Chromatin Fractionation. Chromatin was prepared from rat liver by the method of Marushige and Bonner (22) and treated with diisopropylfluorophosphate (DFP) to inhibit endogenous protease activity (23). Fractionation was carried out as diagrammed in Fig. 1; details of this method have been published previously (21).

Preparation of Chromatin Subunits. Nuclease-resistant subunits of rat-liver chromatin were prepared as follows: DNase II was added to 10 units per A_{260} unit of chromatin in 25 mM sodium acetate (pH 6.6). Digestion was carried out at 24° and was terminated after 90 min by raising the pH to 7.5 with 0.1 M Tris-HCl (pH 11). Nuclease-resistant subunits from chromatin fraction P1 were prepared by homogenizing the pellet fraction in 25 mM sodium acetate (pH 6.6) and redigesting with DNase as described above for whole chromatin. Undigested chromatin (about 20% of the input DNA) was removed by centrifugation at 27,000 $\times g$ for 10-15 min. The supernatant was layered on isokinetic sucrose gradients in SW25.1 cellulose nitrate tubes. The gradients were formed according to Noll (24); the parameters were $C_{TOP} = 15\%$ (weight/volume), $C_{RES} = 34.2\%$ (weight/volume), and $V_{MIX} = 31.4$ ml. All solutions contained 10 mM Tris-HCl (pH 8). Centrifugation was at 25,000 rpm for 36-42 hr. Gradients were analyzed with an ISCO UV Analyzer and chart recorder. Fractions from these gradients were rerun on 5-24% isokinetic sucrose gradients. The parameters were $C_{TOP} = 5.1\%$ (weight/volume), $C_{RES} = 31.4\%$ (weight/volume) and $V_{MIX} = 9.4$ ml. Centrifugation was in the SW 41 rotor at 39,000 rpm at 4° for 16.5 hr.

Subunits were also prepared from chromatin devoid of histone I. Removal of this histone was accomplished by extraction of Virtis-sheared chromatin (45 V, 90 sec) with 0.5 M NaCl at 4°. The resultant nucleohistone was pelleted by centrifugation in the Ti 50 rotor at 50,000 rpm for 18 hr. The pellet was digested with nuclease as described above.

Redigestion of Chromatin Fraction S2. Chromatin of fraction S2 was redigested with nuclease in three different ways: the DNase II present in fraction S2 from the first nuclease treatment was reactivated by adding EDTA to 20 mM and lowering pH to 6.4 with dilute HCl. Alternatively, aliquots of chromatin fraction S2 were dialyzed against either 25 mM sodium acetate (pH 6.6) or 5 mM sodium phosphate (pH 6.7) containing 2.5×10^{-4} M $CaCl_2$ and 2.5×10^{-4} M $MgCl_2$. DNase II was added to the chromatin in sodium acetate buffer to 10 units/ A_{260} unit of chromatin; staphylococcal nuclease was added to the chromatin in sodium phosphate buffer to 50 units/ml. Reactions were carried out at

Abbreviation: DFP, diisopropylfluorophosphate.

* Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England CB2 2QH.

24°. Aliquots were taken at various times to test for the production of trichloroacetic-acid-soluble material (measured by absorbance of the supernatant at 260 nm after centrifugation at 27,000 × g at 4° for 15 min).

DNA Size Estimation. Single-strand DNA lengths were estimated by velocity sedimentation in alkaline sucrose gradients. The parameters for the isokinetic gradients were $C_{TOP} = 15.9\%$ (weight/volume), $C_{RES} = 38.9\%$ (weight/volume), and $V_{MIX} = 6.1$ ml (24). All solutions contained 0.1 N NaOH. Chromatin samples were suspended in 0.1 N NaOH, 2% sodium dodecyl sulfate, 2 M urea, and 100–200 μl aliquots were layered on each gradient. Centrifugation was in the SW50.1 rotor at 48,000 rpm for 16 hr at 20°. DNA molecular weights were determined relative to a standard sized by electron microscopy (320 nucleotide-long, 5.4 S, calf thymus DNA; a gift of Ms. M. Chamberlin). Double-strand lengths were determined in the analytical ultracentrifuge (25).

Analysis of Chromatin Composition. Histone and nonhistone protein content was determined as described (26). Protein was analyzed by sodium dodecyl sulfate-disc gel electrophoresis (27) and by acid-urea gel electrophoresis (28). DNA and RNA were determined by the methods of Schmidt and Tannhauser (29).

Enzymes. DNase II (EC 3.1.4.6) and micrococcal nuclease (EC 3.1.4.7) from *Staphylococcus* were purchased from Worthington.

RESULTS

Chromatin Fractionation. DNase II preferentially attacks a select portion of chromatin DNA. The amount of DNA in this fraction varies depending upon the source of the chromatin, but corresponds quite closely to the measured template activity of the particular chromatin (20). The fractionation scheme used herein is diagrammed in Fig. 1. After 5 min exposure to DNase II, 15% of rat-liver chromatin DNA remains soluble after centrifugation (fraction S1). About 11% of the total DNA is Mg^{++} -soluble and is found in fraction S2. This DNA comprises a subset of whole genomic DNA sequences and is enriched 5-fold in transcriptionally active sequences (21). The DNA has a double-strand length of about 700 base pairs and a single-strand length of 200–600 nucleotides (range of observed values). About 1–3% of this DNA is acid-soluble. After 30 min exposure to DNase II, nearly 80% of the chromatin is found in fraction S1, and 20–24% is found in fraction S2. After prolonged nuclease digestion roughly half of the DNA of both fraction S1 and fraction S2 is acid-soluble. A more detailed description of the kinetics of DNase II action on chromatin is presented elsewhere (J. Gottesfeld, G. Bagi, B. Berg, and J. Bonner, manuscript submitted).

Table 1 lists some of the properties of the chromatin fractions: the composition of fraction P1 is similar to that of unfractionated rat-liver chromatin (21, 22). Fraction S2, however, is enriched in RNA and nonhistone protein and has a reduced content of histone protein. All the major histone species are present in fraction S2 isolated from DFP-treated chromatin. In a previous communication (21) we reported that fraction S2 lacked histone I; the absence of this histone was presumable due to its proteolytic degradation, since the chromatin was not treated with protease inhibitors.

Subunit Structure of Chromatin. Rat-liver chromatin and fraction P1 chromatin have been digested with DNase II for extended periods of time (90 min), and the resulting soluble chromatin has been analyzed by centrifugation in

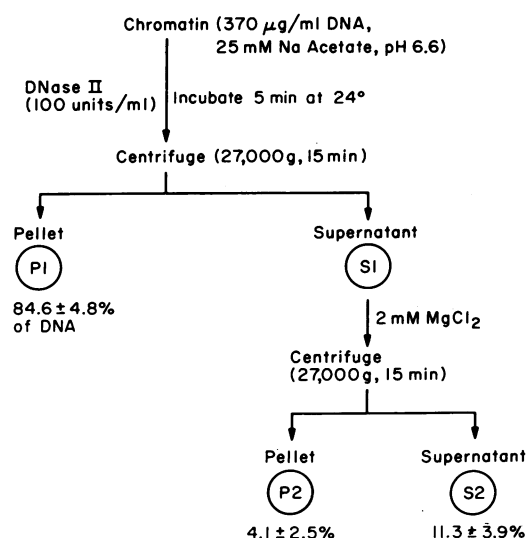


FIG. 1. Fractionation scheme. The yields of DNA in each fraction are the mean and standard deviation for 11 determinations.

isokinetic sucrose gradients (Fig. 2). About 40% of the input DNA sediments extremely slowly; the bulk of this DNA is acid-soluble and hence has been reduced to oligonucleotides by the nuclease. Fig. 2 presents data for whole chromatin depleted of histone I (Fig. 2A) and for P1 chromatin (Fig. 2B). Most of the chromatin sediments at about 11–13 S, with some material sedimenting more rapidly. The properties of nuclease-resistant particles from unfractionated chromatin have been described in detail elsewhere (8–14, 16, 17). We find similar properties for the particles from P1 chromatin: they are composed of equal amounts of protein and DNA (by weight), and the protein complement is almost entirely histone (Table 1). Subunits from native chromatin sediment slightly more rapidly than subunits from histone-I-depleted chromatin (Fig. 2). The sedimentation coefficients (\pm SD) estimated from ten isokinetic 5–24% sucrose gradients were 12.6 ± 0.9 S for subunits of native chromatin and 11.4 ± 0.7 S for subunits of histone-I-depleted chromatin. The length of DNA contained in the subunits has been studied by many workers; values of 120 to 210 base pairs have been obtained

Table 1. Chemical composition of rat-liver chromatin fractions

Sample	Composition relative to DNA (w/w)		
	Histone	Non-histone	RNA
Unfractionated chromatin	1.06	0.65	0.05
P1 chromatin*	1.15	0.58	0.05
11–13S subunits†	1.03	<0.05	—
S2 chromatin*	0.61	1.60	0.25
<i>S2 subfractions</i>			
3–5 S	0.24	0.60	—
14.0 ± 0.8S‡	0.72	1.35	0.3–0.4
18.7 ± 1.0S‡	0.54	3.2	0.3–0.7

* Fractionation carried out as in Fig. 1.

† Similar compositions were obtained for native and histone-I-depleted subunits.

‡ Sedimentation values \pm SD determined from 24 gradients equivalent to those in Fig. 3.

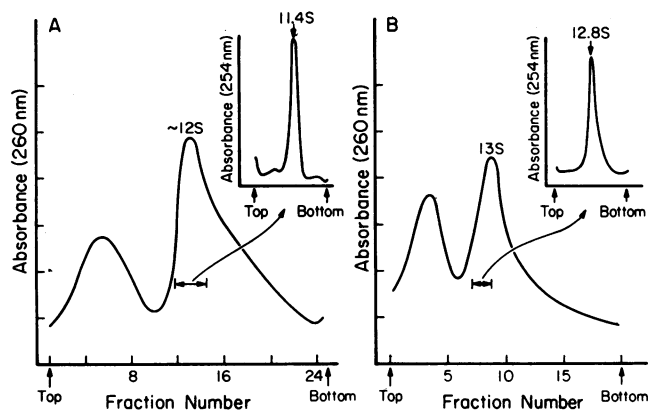


FIG. 2. Sucrose gradient sedimentation of DNase-II-treated chromatin samples. (A) Histone-I-depleted chromatin was digested and soluble chromatin was centrifuged for 42 hr as discussed. (B) Fraction P1 chromatin was digested and soluble chromatin was centrifuged for 36 hr. Fractions were pooled as indicated and rerun on 5–24% gradients (insets).

(10, 13, 14, 16, 17). The 11–13S subunits resemble ν -bodies (2) in the electron microscope (17).

Subunit Structure of Active Chromatin. We now ask whether nuclease-resistant structures occur in transcriptionally active regions of chromatin. Chromatin from rat liver was treated with DNase II for 5 min, fractionated as before (Fig. 1), and S2 material was analyzed on isokinetic sucrose gradients (Fig. 3, curve A). About half of the UV-absorbing material applied to the gradient sediments at 3–5 S; greater than 90% of this material is acid-precipitable after the 5 min nuclease treatment. Two more rapidly sedimenting peaks are seen in the gradient of S2 chromatin, one at 14 S and another at 19–20 S. These gradients were calculated for particles of density 1.44 g/cm³, and so the observed sedimentation coefficients could be in error if the particle densities are very different from 1.4 to 1.5 g/cm³. About 6% of the input nucleic acid pelleted during the centrifugation.

To test whether the 14 and 19S nucleoprotein complexes might be multimers of the 11–13S subunit (i.e., dimers and trimers), chromatin fraction S2 was reincubated with DNase. Upon analysis in isokinetic sucrose gradients (Fig. 3,

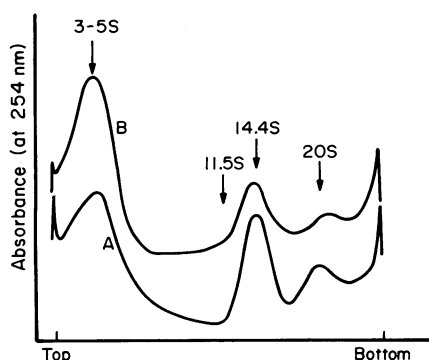


FIG. 3. Sucrose gradient sedimentation of template-active fraction S2 chromatin. Curve A: chromatin was fractionated (Fig. 1) and S2 material was centrifuged for 17.5 hr at 39,000 rpm in 5–24% sucrose gradient. Curve B: fraction S2 was isolated and DNase II reactivated by the addition of 20 mM EDTA (pH 6.4). Incubation was for 1 hr at 24°. The reaction was terminated by raising the pH to 8 with 0.1 M Tris-HCl (pH 11), and the sample was centrifuged as described above.

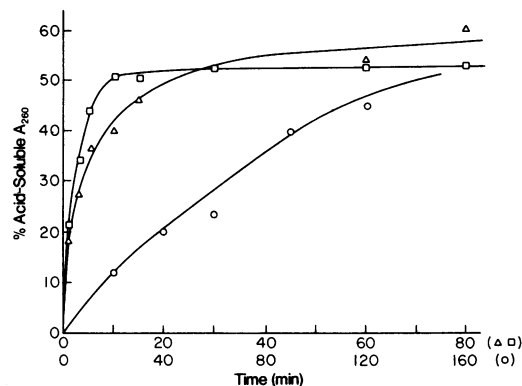


FIG. 4. Kinetics of digestion of chromatin fraction S2. Chromatin was fractionated as described (Fig. 1). S2 chromatin was incubated with nucleases in three ways: reactivation of DNase II (O); addition of fresh DNase II (Δ); addition of staphylococcal nuclease (\square). Aliquots were taken at various times to test for the production of trichloroacetic-acid-soluble material.

curve B) no significant changes were observed in the >10S region. Reincubation with DNase has been carried out in three ways (reactivation of DNase II, addition of fresh DNase II, addition of staphylococcal nuclease), and similar results were obtained with each of these methods. The kinetics of redigestion of S2 chromatin are presented in Fig. 4. With each method of redigestion, about 50–60% of the input A_{260} became acid-soluble. As the reaction approached completion the solutions first became turbid and eventually a precipitate developed.

S2 chromatin has been isolated after various times of nuclease treatment (30 sec to 30 min) and analyzed in sucrose

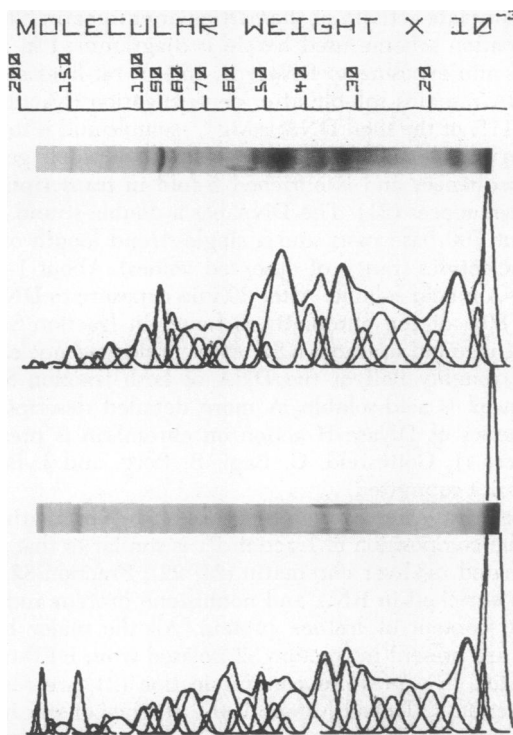


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gels of S2 chromatin subfractions. Total protein of the 3–5S (upper gel) and 14S (lower gel) complexes was electrophoresed as described (27). The stained gels were scanned and the densitometer profiles were resolved into gaussian components by a least-squares computer analysis.

gradients. It was found that the 14 and 19S complexes appear in fraction S2 simultaneously. Thus there is no evidence for a precursor-product relationship between the 19 and 14S complexes. The nuclease-resistant S2 DNA (14 and 19S) was found to have a weight-average single-strand length of 170 nucleotides after the initial DNase treatment (5 min). After prolonged digestion (up to 120 min), the single-strand length was reduced to 120 nucleotides. Nearly all the DNA which sedimented at 3-5 S after the initial DNase digestion was rendered acid-soluble by redigestion.

We have investigated the chemical compositions of the subfractions of S2 chromatin (Table 1). The material at 14-19 S is enriched in both RNA and nonhistone chromosomal proteins. All the major histone species are present in the 14 and 19S complexes; however, these complexes exhibit reduced histone to DNA ratios relative to either unfractionated chromatin or 11-13S subunits (Table 1). The protein population of S2 chromatin has been investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5). Material from the 3-5S (upper gel) and from the 14S (lower gel) position in the sucrose gradient (Fig. 3) has been analyzed. Densitometer scans of the stained gels were resolved into gaussian components by a least squares computer analysis. Numerous quantitative and qualitative differences between the 3-5S and 14S proteins can be recognized in these gels. From the length of DNA contained in the 14S chromatin complex (170 nucleotides), the protein-to-DNA ratio (Table 1), and the complexity of the protein population (Fig. 5), we conclude that the 14S peak consists of a heterogeneous population of nucleoprotein species.

DISCUSSION

The template-active fraction of rat-liver chromatin is organized in a fashion similar to that of inactive chromatin; both fractions consist of regions of nuclease-sensitive and nuclease-resistant DNA. Nuclease-resistant segments in transcriptionally inactive chromatin are due to histone-DNA interactions, while the nuclease-resistant segments of active chromatin are due to DNA complexed with both histone and nonhistone proteins. Nuclease-resistant structures of inactive chromatin sediment at 11-13 S and resemble ν -bodies (2) in the electron microscope (17). The nuclease-resistant complexes of active chromatin sediment at 14-19 S and contain RNA as well as protein and DNA.

Our results shed new light on the findings of Felsenfeld's laboratory (8, 9, 31). These investigators have reported that portions of the globin gene are found in both nuclease-sensitive ("open") and nuclease-resistant ("closed") regions of reticulocyte chromatin. Our results suggest that nuclease sensitivity in a *limit digest* does not discriminate between active and inactive chromatin regions. Felsenfeld's data indicate that regions of the globin genes are always covered with protein (30) but make no distinction between histone and non-histone protein. On the basis of our data we speculate that active genes are complexed with nonhistone as well as histone protein in the 14 and 19S structures.

Inactive genes are complexed mainly with histone and are in the ν -body structures. Although active, like inactive, chromatin contains nuclease-resistant and sensitive regions, there are other major differences. Thermal denaturation and circular dichroism studies (31-33) suggest that active chromatin is in a more extended, more DNA-like conformation than inactive chromatin. The electron microscope has revealed differences in the structure of transcriptionally active and

inactive regions of chromatin. Ribosomal genes in the act of transcription are the length of their transcription product (pre-rRNA) (34). On the other hand, DNA complexed with histones in the ν -body configuration is one-seventh the length of the same deproteinized DNA (4). The basic fiber diameter of inactive chromatin is 100 Å. Active chromatin has a fiber diameter of about 30 Å (35, 36). Thus both physical chemical and electron microscopy studies suggest that DNA of active chromatin is more extended than is the DNA of inactive chromatin. It is probable that this is why active chromatin is more susceptible to nuclease attack. This differential sensitivity to nuclease forms the basis of our fractionation procedure (Fig. 1).

We wish to thank Dr. K. Peters and Mr. W. Wheatley for helpful discussions. This work was supported by the U.S. Public Health Service (GM 86 and GM 13762).

1. Pardon, J. F., Wilkins, M. H. F. & Richards, B. M. (1967) *Nature* **215**, 508-509.
2. Olins, A. L. & Olins, D. E. (1974) *Science* **183**, 330-332.
3. Woodcock, C. L. F. (1973) *J. Cell Biol.* **59**, 368a.
4. Griffith, J. D. (1975) *Science* **187**, 1202-1203.
5. Oudet, P., Gross-Bellard, M. & Chambon, P. (1975) *Cell* **4**, 281-300.
6. Hewish, D. R. & Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 504-510.
7. Burgoyne, L. A. Hewish, D. R. & Mobbs, J. (1974) *Biochem. J.* **143**, 67-72.
8. Clark, R. & Felsenfeld, G. (1971) *Nature* **229**, 101-106.
9. Clark, R. & Felsenfeld, G. (1974) *Biochemistry* **13**, 3622-3628.
10. Axel, R., Melchior, W., Jr., Sollner-Webb, B. & Felsenfeld, G. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 4101-4105.
11. Rill, R. & van Holde, K. E. (1973) *J. Biol. Chem.* **248**, 1080-1083.
12. Sahasrabudhe, C. G. & van Holde, K. E. (1974) *J. Biol. Chem.* **249**, 152-156.
13. Noll, M. (1974) *Nature* **251**, 249-252.
14. Oosterhof, D. K., Hozier, J. C. & Rill, R. L. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 633-637.
15. van Holde, K. E., Sahasrabudhe, C. G., Shaw, B., van Brugger, E. F. J. & Arnberg, A. C. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1365-1370.
16. Senior, M. B., Olins, A. L. & Olins, D. E. (1975) *Science* **187**, 173-175.
17. Gottesfeld, J. M., Kent, D., Ross, M. & Bonner, J. (1975) in *Florida Colloquium on Molecular Biology*, eds. Stein, G. & Stein, J. (Academic Press, New York), in press.
18. Kornberg, R. (1974) *Science* **184**, 868-871.
19. Marushige, K. & Bonner, J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2941-2944.
20. Billing, R. J. & Bonner, J. (1972) *Biochim. Biophys. Acta* **281**, 453-462.
21. Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F. & Bonner, J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2193-2197.
22. Marushige, K. & Bonner, J. (1966) *J. Mol. Biol.* **15**, 160-174.
23. Douvas, A., Harrington, C. & Bonner, J. (1975) *Proc. Nat. Acad. Sci. USA* **72**, in press.
24. Noll, H. (1967) *Nature* **215**, 360-363.
25. Studier, F. W. (1965) *J. Mol. Biol.* **11**, 373-390.
26. Bonner, J., Chalkley, R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C. C., Huberman, J., Jensen, R., Marushige, K., Olenbusch, H., Olivera, B. & Widholm, J. (1968) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12, part B, pp. 3-65.
27. King, J. & Laemmli, U. K. (1971) *J. Mol. Biol.* **62**, 465-477.
28. Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337-346.

29. Schmidt, G. & Tannhauser, S. J. (1945) *J. Biol. Chem.* **161**, 83-89.
30. Axel, R., Cedar, H. & Felsenfeld, G. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 773-783.
31. McConaughy, B. L. & McCarthy, B. J. (1972) *Biochemistry* **11**, 998-1003.
32. Gottesfeld, J. M., Bonner, J., Radda, G. K. & Walker, I.O. (1974) *Biochemistry* **13**, 2937-2945.
33. Polacow, I. & Simpson, R. T. (1973) *Biochem. Biophys. Res. Commun.* **52**, 202-207.
34. Miller, O. L. & Hamkalo, B. A. (1972) in *Molecular Genetics and Developmental Biology*, ed. Sussman, M. (Prentice-Hall, Englewood Cliffs, N.J.), pp. 183-199.
35. Griffith, J. (1970) Ph.D. Dissertation, California Institute of Technology, Pasadena, Calif.
36. Miller, O. L. (1965) *Nat. Cancer Inst. Monogr.* **18**, 79-89.