Structural Aspects of Histone Complexes and Nucleosomes Revealed by the Accessibility of Cysteine Side Chains

Jürgen Bode and Ulrich-Dieter Standt

Gesellschaft für biotechnologische Forschung, Abteilung Molekularbiologie, Braunschweig-Stockheim

Z. Naturforsch. **33 c**, 884–890 (1978); received August 2, 1978

Histone, Chromatin, Protein SH-Reactivity, Nucleosomes

Chemical modification with 5,5′-dithiobis-(2-nitrobenzoic acid) shows that histone H3 cysteines 110 (chicken) or 96 and 110 (calf) are completely protected in native chromatin and core particles but become unmasked simultaneously during a salt induced dissociation. In whole histone extracted from chicken erythrocyte chromatin, H3 Cys-110 residues experience a uniform environment at 2 M NaCl and pH 5.5. In whole histone extracted under the same conditions from calf thymus, H3 Cys-96 provides a more accessible thiol group than Cys-110. At higher pH values a conformational heterogeneity is induced causing a partial exposure of both cysteine side chains. The kinetic approach described in the present work provides a highly sensitive means to probe the homogeneity of H3 containing protein complexes.

Introduction

It has recently been found that the chromatin of simple [1] and higher eucaryotes [2] is organized into a similar subunit structure. Each subunit (nucleosome) comprises 155 – 240 base pairs of DNA which are coiled about a histone core of an octameric structure, *i. e.* $(H4)_2\,(H3)_2\,(H2a)_2\,(H2b)_2$ [3]. The combination of H3 and H4 histones, possessing the most conserved amino acid sequences, provides the major structural determinants of the core particle [4].

While cysteine has been detected in the H4 fraction of echinoderms [5] and in H2b like histones from testis [6, 7], this amino acid generally appears to be restricted to the H3 histone where it constitutes the major contribution (70% in rat thymus) to the total thiol content of the cell nucleus [8]. With the exception of yeast [9], a cysteine in position 110 has been preserved evolutionary. One of the few mutations that have occurred in histone H3 concerns residues 96 which is Ala (60%) or Ser (40%) in pea embryo [10] or an additional cysteine in mammals more advenced than rodents [11].

Little is yet known about the biological role of H3-cysteines and whether they become oxidized during any state of chromosomal activity. It has been claimed earlier that interpolypeptide disulfide bonds contribute to the folding of metaphase chromosomes [12]. Hence, the conservative cysteine residue 110 was expected to be exposed and this assumption seemed to gain support from reactivity studies of the cysteines in calf thymus chromatin towards $(NBS)_2$ [13]. Very recent findings, however, show that Cys 110 sulfhydryls may be buried, *i. e.* in close contact within a single nucleosome as to allow a covalent linkage even between H3 molecules of the same protein core [14].

The aim of present investigation was to clarify whether the conservative cysteine 110 is indeed accessible in chromatin or can be activated reflecting its possible function. It is attempted to assign reactivities towards $(NBS)_2$ of cysteines 110 and 96 by comparing histones and chromatin from chicken erythrocytes and calf thymus. It will be further shown that thiol reactivity is strongly affected by histone-histone interactions and that the kinetic method applied may efficiently be used to probe the conformational homogeneity of complexes containing histone H3. An equivalent information is difficult to obtain by spectroscopic means which may always yield composite data from molecules in a variety of conformations (*cf.* discussion in ref. 15).

Experimental

Materials

Native calf thymus chromatin ($A_{260} = 55.1$; $A_{260}/A_{280} = 1.67$; 1.03×10^{-4} M thiol groups (determined according to Ellman [16])) was obtained as described previously [17] except that the preparation included extensive washings with 0.3 M

Requests for reprints should be sent to Dr. Jürgen Bode, Gesellschaft für Biotechnologische Forschung, Abteilung Molekularbiologie, Mascheroder Weg 1, D-3300 Braunschweig-Stöckheim.

Abbreviations: $PhMeSO_3F$, phenylmethylsulfonyl fluoride; $(NBS)_2$, 5,5′-dithiobis-(2-nitrobenzoic acid); NBS^{0-}, 5-thio-2-nitrobenzoic acid (thiophenolate anion).

NaCl, 0.2 mM $PhMeSO_3F$ to remove thiol containing proteins other than H3. For the preparation of a native whole histone complex, chromatin was exposed to 2 M NaCl, 0.1 M sodium phosphate, pH 7 and freed from DNA by centrifuging at 45 000 rpm for 18 h in a Beckman 50 TI rotor. Alternatively, whole histone was obtained by acid extraction according to Ref. [18] with a recent modification [19] and freeze dried for storage. Histone H3 was isolated according to Johns [20] and purified to a thiol content of 93% of the theoretical value on carboxymethyl cellulose (2.5×20 cm) by stepwise elution with 0.01 M HCl (H2a impurities) and 0.02 M HCl (H3). Histones other than H3 were isolated from whole histone by hydrophobic chromatography [21] on Sepharose containing a C-10 alkyl chain (1.5×40 cm). From this column, histone H1 was eluted by 50 mM sodium citrate of pH 4; the histones H2b, H4 and H2a were then eluted in a sequential manner by 50 mM sodium acetate of pH 4, while H3 stayed bound under these conditions.

The corresponding components from chicken erythrocytes were isolated as follows: 3 l of chicken blood were supplied with 300 ml 0.14 M NaCl, 50 mM EDTA during the collection in a local slaughter house. Erythrocytes were pelleted at $1000 \times g$ and 2 °C for 30 min and were repeatedly washed with and centrifuged from 0.32 M sucrose, 15 mM $MgCl_2$, 0.2 mM $PhMeSO_3F$ under the same conditions. Following the resuspension in 0.14 M NaCl, 5% Triton X-100, nuclei were set free by several bursts from an Ultra Turrax (Janke and Kunkel KG) and pelleted as above. After several washings with the sucrose-$MgCl_2$-$PhMeSO_3F$ medium, the white nuclear pellet was converted to native chromatin ($A_{260} = 61.5$; $A_{260}/A_{280} = 1.69$; 5.7×10^{-5} M thiol groups). Core particles were prepared, essentially as described by Olins *et al.* [22]. The fraction used corresponded to their fraction ν_1 and had an S^0_{20w} value of 11.3 in 100 mM KCl.

Kinetic measurements

A Zeiss PMQ 2 spectrophotometer was linked to a thermostat and connected with a Servogor S recorder. All measurements were performed at 10 °. 2 ml of a solution of 0.1 M sodium phosphate pH 7.0 containing 50 μM histone H3 and various amounts of NaCl were equilibrated together with a corresponding blank which was free of protein. The reference cuvette was used to adjust the photometer to $A_{412} = 0$ and the sample cuvette to zero the recorder. Then equal additions of $(NBS)_2$ (*i. e.* 100 μl of a stock solution containing 12 mg $(NBS)_2$/ml of the corresponding buffer) were made to both cuvettes and the photometer was readjusted to $A_{412} = 0$ as before. At the end of a run, both cuvettes were supplied with 500 μl of a 10 M urea solution for the determination of the final A_{412} value.

Usually, the reference showed a negligible increase of absorbance during a kinetic run; where necessary, corrections for an increased turbidity of the sample were made by separately monitoring the A_{500} values and considering the wavelength dependence of straylight.

Results and Discussion

The relative reactivities of cysteine residues 96 and 110

It has been shown by ^1H NMR spectroscopy [23] and chemical modification studies [24, 25] that the largely hydrophobic carboxyterminal half of histone H3 is the primary site of histone-histone interactions. As cysteines are only located in this segment, a kinetic marker directed to their thiol groups should provide detailed information about particular inter-histone contacts.

It is now well established that the native histone mixture, properly extracted from chromatin at high salt (2 M NaCl) forms structures which are very similar to those in the nucleosome core particle: At neutral or slightly alkaline pH a tetramer or octamer containing one or two species each of the inner histones has been detected [15, 26, 27]. At slightly acidic pH (5.5) and high salt Weintraub *et al.* [27] reported homotypic complex formation between the arginine-rich (H3/H4) and the slightly lysine rich (H2 a/H2 b) histones, respectively.

In order to compare the relative SH reactivities of Cys 96 and 110 in calf thymus or chicken erythrocyte whole histone, we chose the conditions of high salt and pH 5.5. Native chromatin was prepared from *Micrococcus* nuclease digests of chicken erythrocyte and calf thymus nuclei, adjusted to 2 M NaCl, pH 5.5 and the bulk of DNA was removed by ultracentrifugation. The remaining histones had a content of 1 or 2 mol cysteine per 65 000 d of protein, *i. e.* the amount calculated for the respective H3 constituents. These materials were used to

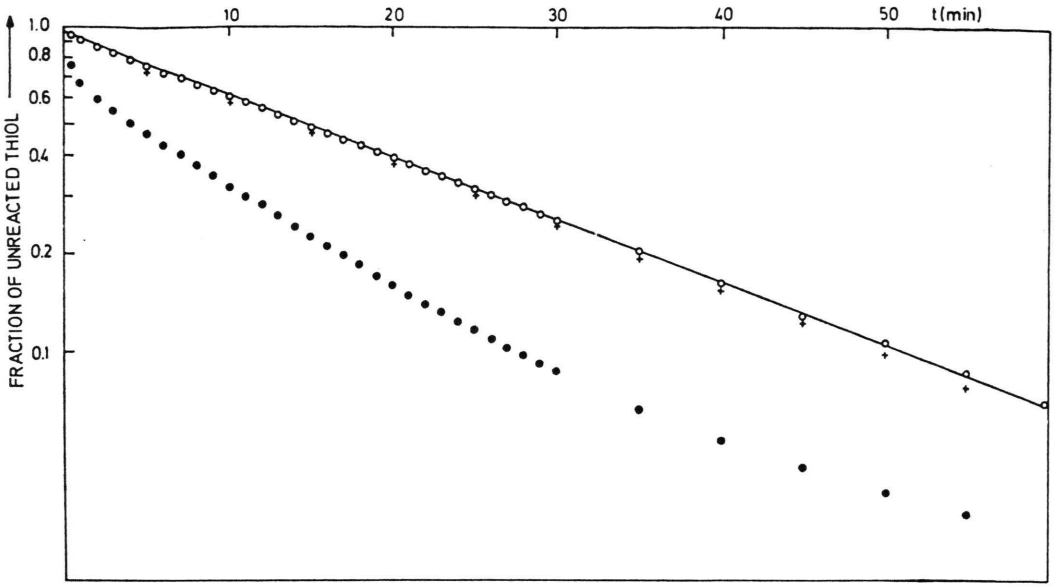

Fig. 1. Correlation of thiol reactivities in whole histone from chicken erythrocytes and calf thymus at pH 5.5. Whole histone from calf thymus (●) and chicken erythrocytes (○) was dissolved in H_2O, placed in acetylated dialysis tubes [28] and dialyzed against the reaction medium (0.1 M sodium phosphates, 2 M NaCl, pH 5.5). For comparison, a run on core particles from chicken erythrocyte chromatin is included (+).

monitor thiol reactivities and the results are shown in Fig. 1. The same experiments were performed with a preparation of core particles, which contain the four inner histones besides a 140 base pair segment of DNA and with acid extracted histones which had thoroughly been reconstituted (slow increase in ionic strength by dialysis). Interestingly, almost superimposable time courses were found in all three cases which were clearly distinct, however, from acid extracted histones used without reconstitution (not shown).

If our data are presented in a semilogarithmic manner (Fig. 1); reaction at Cys 110 (chicken) is seen to proceed in accord with a single rate constant ($k = 29\ \text{min}^{-1}\ \text{M}^{-1}$ *cf.* top trace). A single reactivity for a distinct cysteine is obviously highly indicative of a conformational homogeneity. On the other hand, calf thymus histones yield a nonlinear time course as expected for nonequivalent cysteines (bottom trace): a very fast component ($k = 490\ \text{min}^{-1}\ \text{M}^{-1}$) is apparent besides reactivities in the range observed with the chicken histones. If one accepts that a unique conformation has been established as in the previous sample, this finding must mean that of the two cysteines of calf thymus histone H3, Cys 96 provides the more reactive thiol group, whereas Cys 110 reacts more slowly like that in chicken histone H3. We could substantiate this assumption by a reproduction of the respective time course using histone H3 from calf thymus which had been supplemented with the H4 fraction or all the residual histones from chicken (not shown).

The previous assignment of Cys 110, as belonging to the high reactivity class [13, 29] was found to be based upon simple model considerations on an isolated H3 molecule, *i. e.* the probability of Cys 96 or 110 to be located in an α-helix or coil segment was estimated from the nature of the neighbouring residues [13, 30] and location in a coil segment was assumed to decrease reactivity. These considerations did not take into account any mode of self- or cross-interaction. Isolated histone molecules with an elaborate secondary structure may exist at 0.05 – 0.1 M NaCl but they are of no biological significance. Our data suggest that distinct microenvironments for the cysteines are a property of the native complex at pH 5.5 and for this case assignments should be reversed.

More complicated complexes involving histone H3

At pH 6.8 – 7.0, calf thymus whole histone displays an almost 1:1 ratio between the slow and fast reacting set of thiols. Formerly, this has suggested to others [13] and to us [19] distinct re-

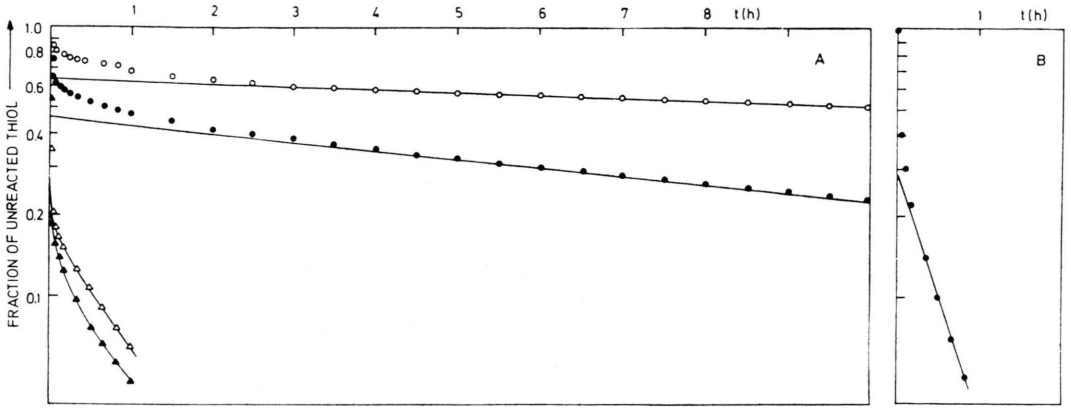

Fig. 2. Conformational heterogeneity revealed by thiol reactivities at pH 7. A) Whole histone from calf thymus (●) and chicken erythrocytes (○) was reconstituted as above by dialysis against 0.1 M sodium phosphate, 2 M NaCl, pH 7.0. ▲, △ composition of the fast phase of the reaction, calculated by subtracting the extrapolated line (*i. e.* ordinate values of "—") from the observed time course (●, ○), *cf.* ref. 31. B) Purified histone H3 from calf thymus was treated and reacted as the whole histones in Fig. 2 A.

activities for cysteines 96 and 110. The extension of our work to histones from chicken erythrocytes now offers the opportunity to reduce the complexity of the system by observing the behaviour of cysteine 110 alone (Fig. 2). Surprisingly, neither chromatin nor core particles or a reconstituted mixture of histones was apt under these conditions to yield a homogeneous reaction. In fact, the only obvious difference between comparative runs on the histones from calf and chicken was a relative increase of the fast component in the former case. We therefore conclude, that at pH 7, the thiols at positions 96 and 110 are both contributing to the fast and slow phase of the reaction, respectively.

An even more striking feature of the reaction at pH 7 is an enormeous retardation of the over-all kinetics if compared with pH 5.5 (note the different time scales in Figs 1 and 2). This trend is opposed to the normal pH-effect [32] indicating that rate constants are not governed by the degree of thiol deprotonation but rather respond to steric factors. In a separate reconstitution experiment we were able to demonstrate that the relative inaccessibility of the cysteines reflects the occupancy of the H3 binding domains by two different histones, *i. e.* histones H4 and H2 a were necessary to mimic the traces in Fig. 2 A (note the time course in Fig. 2 B for pure histone H3). This is in accordance with the concept of a "heterotrypic tetramer" (H2a-H3-H4-H2b) at pH 7 derived by Weintraub [27], with specific contacts of H3 towards H4 and H2a.

Detection of stable conformational constituents in a complex histone mixture

In Fig. 2 A we have applied the method of successive substractions [31] in order to resolve the composite kinetics into its constituents. It is seen that there are about 50% of slowly reacting thiols if two cysteines are present (calf thymus histones, *cf.* ref. 13) and 65% if residue 110 of H3 is the only cysteine (chicken erythrocyte histones). In both cases, the fast phase of the reaction in itself is heterogeneous.

We have carried out a large number of corresponding analyses under a variety of ionic conditions. Our results are summarized in Fig. 3 which reflects the fraction of slowly reacting thiols dependent upon the ionic strength. The comparison between calf thymus histone H3, whole histone from calf thymus and chicken erythrocytes shows a remarkable constancy in the distribution between fast and slowly reacting thiols if complete histone mixtures are used, in contrast to histone H3 alone where this parameter is a continuous function of ionic strength (*cf.* also ref. 32). It is our conclusion that in deficient mixtures a continuous shift occurs between various nonspecific aggregation states while the majority of a core histone mixture assembles to form a stable low reactivity complex which endures all but the very lowest ionic strength. This stable complex may be the heterotypic histone tetramer described by Weintraub *et al.* [27].

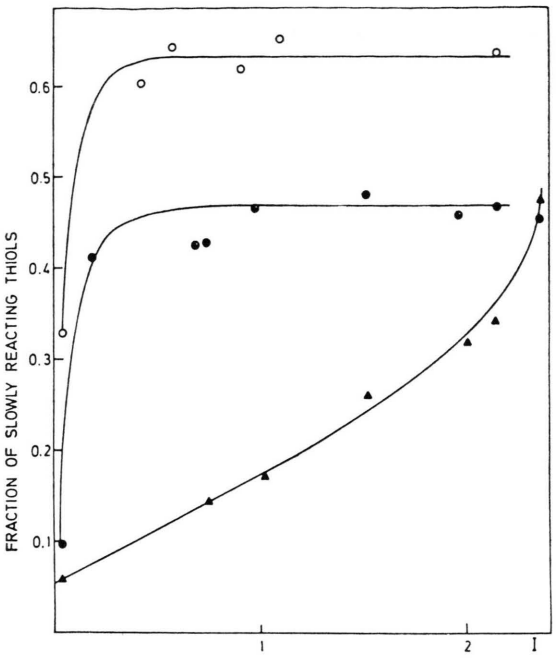

Fig. 3. Effect of ionic strength (I) upon the fraction of slowly reacting thiols at pH 7. The distribution between the fast and slowly reacting set of thiols was determined as in Fig. 2. Data published by Hyde and Walker [13] have been replotted correspondingly and are added for comparison (⊕). ▲, calf thymus histone H3; ●, ⊕ calf thymus whole histone; ○, chicken erythrocyte whole histone.

What are the reasons for an involvement of histone H3 in more than one complex at pH 7? In principle, we could have to deal with two or more subfractions of H3 as observed in calf thymus [33] and pea embryo [10]. In this case however, one would expect a similar heterogeneity for the complex at pH 5.5. It therefore appears conceivable that those 65% H3 molecules which react slowly (Fig. 3, top trace) have met interacting partners at pH 7 with which they associate to a stable complex. The remaining 35% would then be engaged in various low stability aggregates (*cf.* the heterogeneity of the fast phase in Fig. 2 A). Our observation that complex mixtures arise at pH 7 but not pH 5.5 (*cf.* Fig. 1 and 2 A) could therefore mean that heterogeneity is brought about by modified (phosphorylated, acetylated or methylated) protomers of H2a or H2b, *i. e.* histones not associated with H3 at lower pH value.

On the thiol accessibility in native chromatin preparations

Both cysteines of calf thymus histone H3 are located in a nonbasic region of 31 amino acid residues (84 – 114) which in the nucleosome is part of a compact protein core. The NH_2 terminal segment 1 – 83 is very basic and has the highest potential for an interaction with DNA. There are other clusters of basic amino acids beyond residue 118 which may also be involved in binding of DNA phosphates [34]. These considerations may lead to the prediction that thiol accessiblity should be unaffected by the presence of DNA but this matter has remained controversial:

Exposure of nucleosomes from trout testis to a 20-fold excess of *p*-hydroxymercuribenzoate in 10 mM Tris-HCl yields only little reaction at the single H3-cysteine in position 110 [35]. The corresponding residue in calf thymus nucleohistone (solubilized by shearing), however was reported to be freely acessible to $(NBS)_2$ in 0.7 mM sodium phosphate, while Cys 96 appeared to be buried below 0.7 M NaCl 13. In our hands, titrations of native chromatin or core particles had consistently led to at most a minor increase of the A_{412} value which was fully accounted for by the development of some turbidity. A reinvestigation of these experiments (performed at pH 7) yielded the results depicted in Fig. 4: No incorporation of NBS occurs into native calf thymus chromatin below 0.2 M NaCl. The insoluble nucleohistone existing between 0.14 and 1 M NaCl shows progressively more reaction as the upper limit is approached. We noted a quantitative conversion of both thiols be-

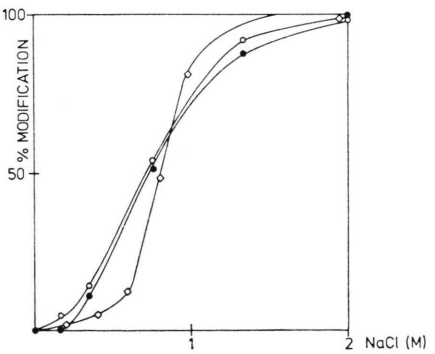

Fig. 4. Influence of DNA upon thiol reactivities. Nucleoprotein (A_{260}=55) was adjusted to 0.02 M sodium phosphate pH 7.0 or 5.5 by dialysis. 3 ml portions were supplied with solid NaCl to the indicated concentration and $(NBS)_2$ was added to a ratio of 27 mol/mol H3. Maximal modification was determined in solutions containing 2 M NaCl, 4 M urea. All samples were left for 4 days at 4 °C before A_{412} values were read. —●—, —○—, calf thymus or chicken erythrocyte chromatin, modified at pH 7; prior to analysis, the samples were clarified by centrifugation. —◇—, core particles from chicken erythrocyte chromatin, modified at pH 5.5; direct measurements.

tween 1 and 2 M NaCl where solutions become less turbid due to the dissociation of the components. Chicken erythrocyte chromatin displayed a related ionic strength dependence and it is hence believed that Cys 110 and Cys 96 become exposed simultaneously rather than in a sequential manner. Qualitatively similar results were also obtained with core particles which (due to the absence of histones H1 and/or H5) offered the advantage of being soluble at any ionic strength making possible a direct colorimetric observations.

Conclusions

In the physiologically quiescent chromatin of chicken erythrocytes as well as in interphase chromatin derived from calf thymus, H3-thiol groups are found to be buried at physiological ionic strength. This result also pertains to core particles from both sources which are devoid of the so called "linker regions", including histones H1 and H5. If cysteines 96 or 110 become accessible during any phase of the cell cycle, their exposure must be triggered by histone modification or another structural alteration of the chromatin subunit. It has recently been speculated that a specific H3-phosphorylation occuring just before metaphase might bring about the rearrangements required [36].

To obtain an incorporation of NBS-groups into the H3 fraction, the electrostatic interactions between DNA and histones had to be reduced by the addition of salt. At 2 M NaCl, pH 5.5, cysteine residues 110 experience a homogenous environment and in this medium Cys 96 is the more reactive residue. Under other conditions, both cysteines may be present in conformations which render them activated or unreactive.

Based on these observations, we suggest that the kinetic approach described may be utilized as a highly sensitive tool to probe conformational homogeneity and to detect stable components in composite histone aggregate mixtures. While the application of $(NBS)_2$ is restricted to thiol concentrations exceeding 10^{-5} M, the analysis scheme may be extended to about 5×10^{-8} M thiol using a fluorogenic marker with thiol specificity. Studies of this type are now in progress in our laboratory.

We are very grateful to Prof. K. G. Wagner and Dipl. Chem. E. Wingender for their interest and many stimulating discussions, to Dr. H.-A. Arfmann for an introduction to hydrophobic chromatography. We also thank Mrs. R. Jähne and Miss M. Kuntz for their help and the Geflügelzucht R. Bordan for providing facilities for the collection of chicken blood. This work was supported by the Deutsche Forschungsgemeinschaft (Wa 91), the Fonds der Chemischen Industrie, and a grant from Friedrich-Ebert-Stiftung.

[1] J. O. Thomas and V. Furber, FEBS Letters **66,** 274–280 (1976).
[2] R. D. Kornberg, Science **184,** 868–871 (1974).
[3] J. O. Thomas and R. D. Kornberg, Proc. Nat. Acad. Sci. USA **72,** 2626–2630 (1975).
[4] M. Bina-Stein and R. T. Simpson, Cell **11,** 609–618 (1977).
[5] J. A. Subirana, FEBS Letters **16,** 133–136 (1971).
[6] V. Monesi, Exp. Cell Res. **36,** 683–691 (1964).
[7] A. Shires and M. P. Carpenter, J. Biol. Chem. **251,** 4155–4158 (1976).
[8] H. Deakin, M. G. Ord, and L. A. Stocken, Biochem. J. **89,** 296–304 (1963).
[9] W. F. Brandt and C. von Holt, FEBS Letters **65,** 386–390 (1976).
[10] L. Patthy, E. L. Smith, and J. Johnson, J. Biol. Chem. **248,** 6834–6840 (1973).
[11] S. Panyim, K. R. Sommer, and R. Chalkley, Biochemistry **10,** 3911–3917 (1971).
[12] A. Sadgopal and J. Bonner, Biochem. Biophys. Acta **207,** 227–239 (1970).
[13] J. E. Hyde and I. O. Walker, Nucleic Acids Res. **1,** 203–215 (1974).
[14] R. D. Camerini-Otero and G. Felsenfeld, Proc. Nat. Acad. Sci. USA **74,** 5519–5523 (1977).
[15] R. D. Kornberg, Ann. Rev. Biochem. **46,** 931–954 (1977).
[16] G. L. Ellman, Arch. Biochem. Biophys. **82,** 70–77 (1959).
[17] J. Bode, L. Willmitzer, and K. Opatz, Eur. J. Biochem. **72,** 393–403 (1977).
[18] Y. Kobayashi and K. Iwai, J. Biochem **67,** 465–472 (1970).
[19] J. Bode and K. G. Wagner, Biochem. Biophys. Res. Commun. **62,** 868–876 (1975).
[20] E. W. Johns, Biochem. J. **92,** 55–59 (1964).
[21] H.-A. Arfmann and S. Shaltiel, Eur. J. Biochem. **70,** 269–274 (1976).
[22] A. L. Olins, R. D. Carlson, E. B. Wright, and D. E. Olins, Nucl. Acids Res. **3,** 3271–3291 (1976).
[23] T. Moss, P. D. Cary, C. Crane-Robinson, and E. M. Bradbury, Biochemistry **15,** 2261–2267 (1976).
[24] P. N. Lewis, Can. J. Biochem. **54,** 963–970 (1976).
[25] P. N. Lewis, Biochem. Biophys. Res. Commun. **68,** 329–335 (1976).
[26] S. Y. Chung, W. E. Hill, and P. Doty, Proc. Nat. Acad. Sci. USA **75,** 1680–1684 (1978).
[27] H. Weintraub, K. Palter, and F. van Lente, Cell **6,** 85–110 (1975).

[28] L. C. Craig, Methods in Enzymology (C. H. W. Hirs, ed.), **Vol. 11,** pp. 870–905 (1967).
[29] J. E. Hyde and I. O. Walker, Biochem. Biophys. Acta **490,** 261–271 (1977).
[30] J. Palau and E. Padros, FEBS Letters **27,** 157–160 (1972).
[31] W. J. Ray and D. E. Koshland, J. Biol. Chem. **236,** 1973–1979 (1961).
[32] J. Palau and J. R. Dabán, Eur. J. Biochem. **49,** 151–156 (1974).
[33] W. F. Marzluff, L. A. Sanders, D. M. Miller, and K. S. McCarty, J. Biol. Chem. **247,** 2026–2033 (1972).
[34] J. E. Hyde and I. O. Walker, Nucl. Acids Res. **2,** 405–421 (1975).
[35] P. Maher and E. P. M. Candido, Can. J. Biochem. **55,** 404–407 (1977).
[36] L. R. Gurley, R. A. Walters, and R. A. Tobey, J. Cell Biol. **60,** 356–364 (1974).