Volume 3 no.12 December 1976 Nucleic Acids Research

Chromatin structure: a property of the higher structure of chromatin and the time course of its formation during chromatin replication

L.A.Burgoyne, J.D.Mobbs and A.J.Marshall

School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia 5042.

Received 13 September 1976

ABSTRACT

The action of a number of enzymes and metals on one nuclear preparation were interpreted in terms of the existence of a fragile but highly DNAase-I resistant feature of chromatin superstructure. The generation of this DNAase-I resistance feature of chromatin was then followed during normal DNA synthesis in the regenerating rat liver by following the disappearance of a transitory DNAase-I susceptible state. This transitory, DNAase-I susceptible state appears to be extremely similar to the post-synthetic, DNAase-I susceptible state that has been described in HeLa[32].

INTRODUCTION

The rat liver nuclear preparations. The preparation procedure used in this paper excludes divalent metals and uses polyamines as stabilizers[1] and it appears to retain the extremely high molecular weight of natural DNA although this does rapidly decrease on exposure to magnesium with trace calcium[1,25]. The full complement of histones is present and autoproteolysis of the nuclear histones appears to be acceptably low[2]. The nucleoprotein has the characteristic repeating chromatin substructure and one internal nuclease that cleaves at regularly placed sites[2,3,4]. It is however highly resistant to the action of a number of cytoplasmic nucleases[5].

The preparation appears to have lost many of the enzymes associated with normal nuclear metabolism, e.g. ligase and the S-phase DNA polymerase, and will not carry out normal DNA replication[26,27]. The thorough loss of these macromolecules strongly implies that the nuclear membrane is, if present, not an effective barrier to the exchange of most macromolecules.

Thus this nucleus appears to be some sort of core, or stable basal structure, that has had leached or stripped from it many of the more loosely associated substances.

A number of nucleases and proteases have been used to study chromatin organisation at the level of its basic repeating unit. In this paper we

wish to consider the deductions that can be made about higher orders of structure from a study of the action of the following three enzymes on this one nuclear preparation.

Effects of trypsin on chromatin structure. This enzyme rapidly destroys histone H1 and modifies other nuclear histones[18,19,20]. Histone H1 is thought to be external to the chromatin subunit so that its destruction does not disrupt the subunit structure[11,12,13,14]. However, H1 has been implicated in the formation of higher order chromatin structures[11,15,16] and its removal results in extensive unfolding of chromatin[21,22].

Effects of Ca-Mg endonuclease on chromatin structure. The endogenous Ca-Mg endonuclease of rat liver readily cleaves sites regularly spaced along the repeating chromatin substructure but it does not release large amounts of nucleotides or oligonucleotides[2,3,4] and even when its action is approaching completion it does not induce gross solubilization of chromatin or release large amounts of DNA from nuclei[2].

Effects of micrococcal nuclease on chromatin structure. This first attacks the same zones or sites that the Ca-Mg endonuclease attacks but it seems to much more readily convert the DNA in these zones to acid-soluble fragments[2,6,7] than the Ca-Mg endonuclease does.

Effects of DNAase-I on chromatin structure. This enzyme appears to attack a much wider range of sites on the chromatin substructure than either of the previous two nucleases and this aspect of its action has been the subject of a number of studies[6,9,29,32]. It also rapidly produces acid soluble fragments from DNA that is exposed to it. However, unlike the other three enzymes, the action of DNAase-I now appears to be highly restricted by the higher order coiling or packing of the regular chromatin substructure. This hindrance of DNAase-I and its structural significance is the main subject of this paper.

METHODS

Labelling rat livers. Two-thirds of the liver was removed surgically from 150 gm, 6-week old, female, Hooded Wistar rats. After 24 hr the regenerating tissue was labelled by intraperitoneal or intraportal injection of ^{3}H-thymidine. Intraportal injections were used for the short labelling time experiments and they consisted of 150 μCi Me-^{3}H-thymidine, 16 Ci/mmol, in 0.5 ml of 0.1 M NaCl infused over a period of approximately 1.5 min.

Termination of labelling and preparation of nuclei. Labelling periods were measured from the time the label infusion began to the time the liver was crushed between 2.0 cm thick plates of iced ($0^{o}C$) aluminium.

The crushed tissue from each liver was then homogenized with carrier liver tissue from three normal unlabelled rats and the labelled nuclei and carrier nuclei prepared together[1]. This preparation procedure excludes divalent metals and stabilises the nuclei with polyamines.

The standard nuclear-digestion. Nuclear densities were measured in terms of nuclear DNA by the Burton[24] method. The standard incubations were all in Buffer A[1], 10^{-4} M EDTA, 0.34 M sucrose, 9 mM $MgCl_2$, pH 7.5. EGTA[31] was added as an Na^{+}-EGTA-Mg^{++} complex, pH 7.5, 2 mM final concentration with respect to both Mg^{++} and EGTA.

Estimation of acid soluble fragments produced during digestions. 0.05 ml samples of nuclear suspensions were removed at intervals of 10 min and placed in 0.25 ml lots of cold 6.64% trichloroacetic acid. After centrifugation ($0^{o}C$, 30 min, 10,000 g), 0.2 ml of the supernatant was neutralised, dried in vacuo, redissolved in water and counted. These counts were then expressed relative to the total counts in the nuclei. The total counts in the nuclei were estimated by counting tritiated water produced by oxidation of samples of nuclei. Appropriate internal standards allowed corrections for the differential efficiencies and quenching of the two different counting methods.

Enzymes. DNAase-I. Calbiochem B Grade. Activity checked in a standard assay mix that consisted of 0.04 M tris-HCl, pH 7.4, 0.2×10^{-4} M EDTA, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, calf thymus DNA to $E_{260} = 0.68$. One unit of activity was defined as the amount of DNAase that takes one minute to give half the hyperchromic shift in one ml of the assay mix.

The nuclear digests reported in this paper all contained 140 of these units per ml corresponding to approximately 40 µg/ml of DNAase-I (by weight) and 0.064 E_{280} optic density units of DNAase protein.

Some batches of DNAase-I were treated with phenyl methyl sulphonyl fluoride to inhibit traces of contaminating proteases.

Trypsin-TPCK from Worthington. Its final concentration in the nuclear digests was 67 µg/ml protein.

Micrococcal nuclease from Worthington. Its final concentration in nuclear digests was 400 of the manufacturers units per ml.

RESULTS

The resistance of normal, nuclear DNA to DNAase-I and methods of breaking this resistance. All experiments in this section used nuclei from rats that were labelled by intraperitoneal injection 24 hr after hepatectomy. The rats were then used 7 days after the injection to ensure that most chromatin was mature, interphase chromatin.

Figure 1 shows the resting chromatin's resistance to DNAase-I and how

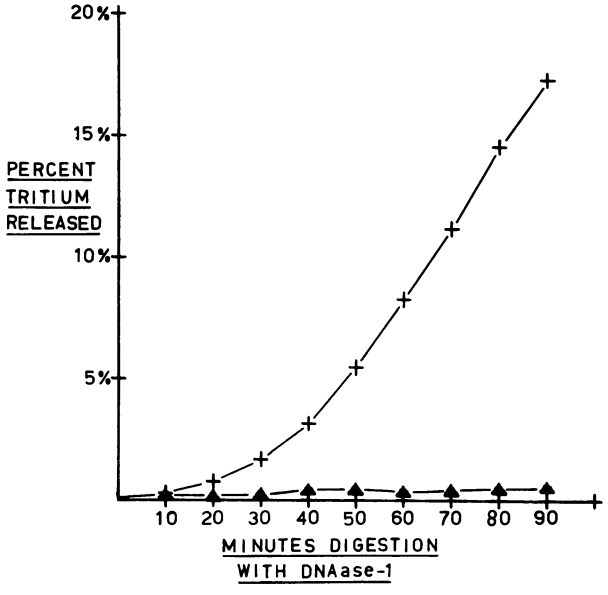

FIGURE 1. THE RESISTANCE OF NUCLEAR DNA TO DNAase-I AND ITS EXPOSURE BY TRYPSIN.

One rat liver was labelled with ^{3}H-thymidine by intraperitoneal injection, 24 hr after hepatectomy. Nuclei were prepared seven days after labelling. Two incubation mixes, 1 ml each, were prepared containing DNAase-I. One of these mixes also contained trypsin.

All preparations, incubations, sampling procedures and measurements of ^{3}H release were by the standard methods. The nuclear density was 2,500 µg nuclear DNA/ml. The ^{3}H content was 73,300 cpm/ml.

The curves show the release of acid soluble ^{3}H expressed as a percentage of the total radioactivity present in the nuclei.

▲ - Standard incubation with DNAase-I only.

\+ - Standard incubation with DNAase-I plus trypsin.

trypsin breaks this resistance. The two digestions (+ and - trypsin) shown in Figure 1 were carried out on one batch of nuclei under the standard conditions in the presence of EGTA and free Mg^{++}.

Figure 2 shows the results of an experiment in which another batch of nuclei was divided into two lots. One lot was digested in the presence of magnesium and EGTA, as before, but the other lot had 1 mM free Ca^{++} instead of the EGTA. The first lot shows, again, the high stability of the resting chromatin exposed to DNAase-I but it is evident that the Ca^{++} has also had a powerful destabilizing effect.

As it is known that calcium strongly stimulates the action of DNAase-I [23] an attempt was made to differentiate this potential explanation

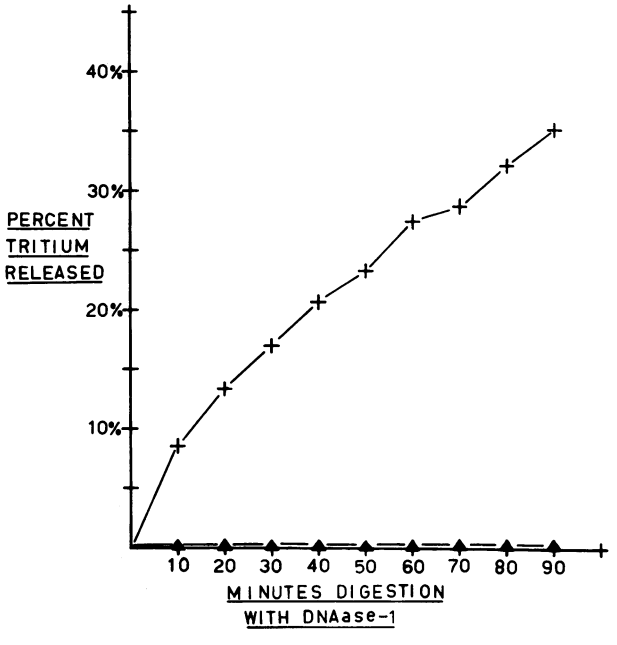

FIGURE 2. THE RESISTANCE OF NUCLEAR DNA TO DNAase-I AND ITS EXPOSURE BY CALCIUM.

Two standard incubation mixes containing labelled nuclei and DNAase-I were prepared as in Figure 1. However, in one of the mixes the Na^{+}-EGTA-Mg^{++} complex was omitted and replaced by 1 mM $CaCl_2$. The DNAase-I used in this experiment had been treated with phenyl methyl sulphonyl fluoride.

The nuclear density was 3,400 µg nuclear DNA/ml. The ^{3}H content was 260,000 cpm/ml.

The curves show the release of acid soluble ^{3}H expressed as a percentage of the total radioactivity present in the nuclei.

▲ - Standard incubation with DNAase-I only.

\+ - As for standard incubation except minus EGTA-Mg^{++} and plus Ca^{++}.

of the calcium effect from an explanation in terms of the nuclear Ca-Mg endonuclease. Thus preincubation experiments with the divalent metals were attempted but they had to be abandoned because preincubation and rewashing procedures, even in the absence of divalent metals, increased the susceptibility of the nuclei to DNAase-I. However, these experiments did, at least, indicate how readily the nuclear structure may be made susceptible to the action of DNAase-I by handling procedures.

Examination of bulk DNA molecular weight during DNAase-I digestion. A batch of labelled but resting nuclei was digested with DNAase-I as in Figure 1. After 90 min digestion the release of acid soluble fragments was compared to the average molecular weight of the remaining DNA.

The mean size of the undigested DNA was estimated on 2.5% acrylamide gels[3] and was found to be greater than the gels could resolve, i.e. greater than approx. 800 base pairs. The corresponding release of acid soluble fragments during this digest was 0.5% of the total DNA.

This experiment showed that the very low rates of release of acid soluble fragments observed in this type of digestion are not being accompanied by an extensive fragmentation of the genome as is the case with the endogenous Ca-Mg endonuclease[2].

Demonstration of the existence of a transitory, DNAase-I unstable state of chromatin during chromatin synthesis. A series of experiments were carried out with the same basic design. Rats were hepatectomised and then, 24 hr later, a pulse of ^{3}H-thymidine was infused into the portal vein. Then, at intervals between 3.2 and 10 minutes after the beginning of the pulse, the livers were used to prepare nuclei by the standard procedure.

Each batch of nuclei had one lot exposed to DNAase-I in the presence of trypsin and one lot exposed to DNAase-I in its absence. The results are shown in Figure 3 and summarised in Figure 4.

It is clear that the newly synthesized chromatin was highly susceptible to DNAase-I and this susceptibility decreased to very low levels over the time span of 10 min post-pulse. At 3.2 min the newly labelled chromatin had a susceptibility to DNAase-I that is comparable to that of trypsinized chromatin but by 10 min post-pulse the slight remaining susceptibility may be only due to small amounts of post-pulse labelling.

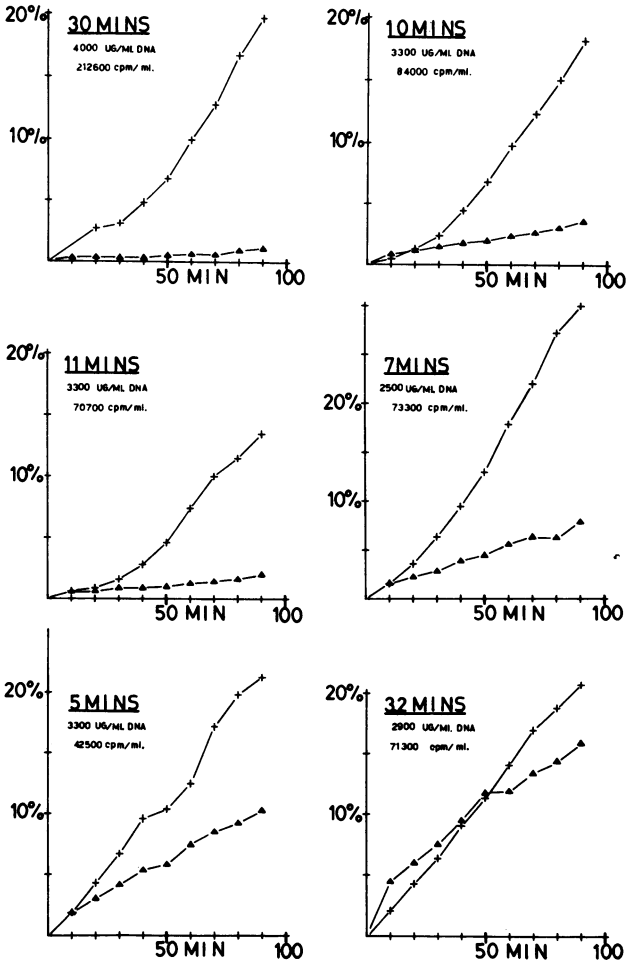

FIGURE 3. THE MATURATION OF NEWLY SYNTHESIZED CHROMATIN TO A DNAase-I RESISTANT STATE.

Each graph represents an experiment in which one rat was pulse labelled with ^{3}H-thymidine, intraportally, and after a labelling period between 3.2 and 10 minutes the nuclei were prepared and digested with DNAase-I or DNAase-I plus trypsin.

The curves show the release of acid soluble ^{3}H expressed as a percentage of the total radioactivity present in the nuclei.

▲ - Digests that contain DNAase-I only.

\+ - Digests that contain DNAase-I plus trypsin.

Digestion mixes all contained the standard amounts of enzymes. All assays were in the presence of the standard amounts of Na^{+}-EGTA-Mg^{++}, free Mg^{++} and other buffer ions. The concentrations of nuclei and radioactivity in each experiment are shown on the diagrams. In all experiments the release of acid soluble ^{3}H in the absence of DNAase-I was too low for this estimation procedure to measure.

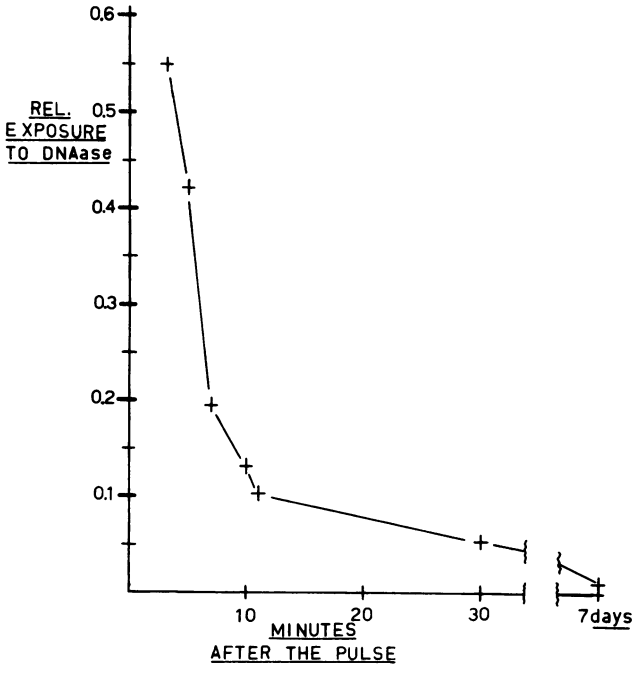

FIGURE 4. THE TIME COURSE OF PROTECTION OF NEWLY SYNTHESIZED DNA FROM THE ACTION OF DNAase-I.

This figure is a summary and rearrangement of the results shown in Figure 3 and Figure 1. For each pair of digests, in these figures (DNAase-I, + and - trypsin) the average rate of release of acid soluble ^{3}H due to DNAase-I alone is expressed as a proportion of the rate of release of acid soluble ^{3}H in the presence of trypsin as well as DNAase-I, i.e.

$$\text{(Relative exposure to DNAase-I)} = \frac{\text{(Rate with DNAase-I)}}{\text{(Rate with DNAase-I plus trypsin)}}$$

This ratio was then plotted against the post-pulse time for each experiment.

The average rates of release of acid soluble ^{3}H were all taken as the average rate between the 50 min and 90 min digest times.

The absence of any resistance of resting chromatin to the action of micrococcal nuclease. Figure 5 shows the results of an experiment in which resting chromatin that had been labelled for 7 days was exposed to micrococcal nuclease in the presence and in the absence of trypsin.

It can be seen that the chromatin appeared to be almost as susceptible to the micrococcal nuclease in the absence of the trypsin as in its presence.

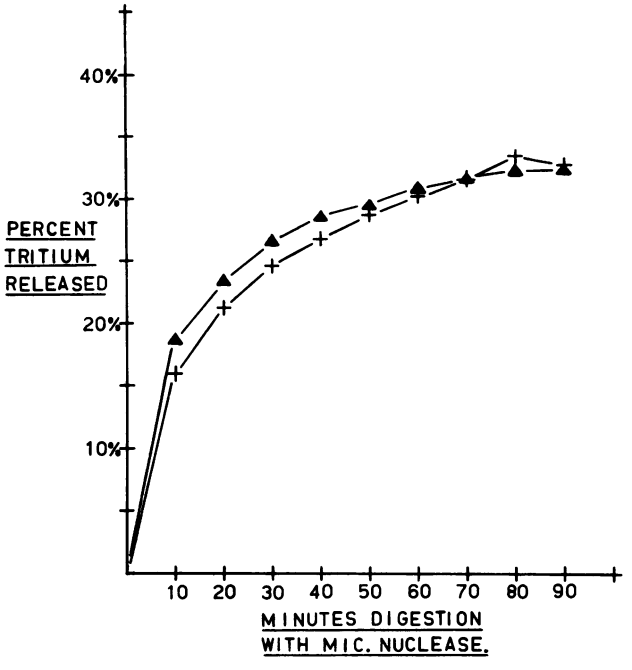

FIGURE 5. UNDER THE STANDARD CONDITIONS MICROCOCCAL NUCLEASE ATTACK IS RAPID AND TRYPSIN INDEPENDENT.

Two standard 1 ml incubation mixes containing labelled nuclei were prepared with the following modifications. Calcium was present at 1 mM. The free Mg^{++} and the Na^{+}-EGTA-Mg^{++} complex was omitted. The nuclease present was micrococcal nuclease (400 units/ml). The nuclear density was 2,300 µg nuclear DNA/ml. The ^{3}H content was 270,000 cpm/ml.

One of the two assay mixes also contained the standard amount of trypsin.

The curves show the release of acid soluble ^{3}H expressed as a percentage of the total radioactivity present in the nuclei.

▲ - Micrococcal nuclease plus trypsin.

\+ - The mix with micrococcal nuclease alone.

This forms a marked contrast to the results obtained with DNAase-I. However, it should be noted that calcium is an unavoidable co-factor in micrococcal nuclease digestions.

DISCUSSION

The resistance of intact nuclear DNA to nucleases seems to be almost an all-or-none phenomena. The resting nucleoprotein is highly resistant to DNAase-I but the newly synthesized nucleoprotein is highly susceptible.

However, trypsin attack converts the resting chromatin from a highly resistant state to a highly susceptible state. In striking contrast to this, micrococcal nuclease is very active on intact chromatin and trypsin has virtually no effect on its action. Calcium in the presence of magnesium or, perhaps, calcium alone also converts the chromatin from a highly DNAase-I resistant structure to a highly DNAase-I susceptible structure. Finally, newly synthesized chromatin is very DNAase-I susceptible but rapidly becomes completely resistant.

These results, together, suggest the presence of a very definite superstructure for chromatin that is highly DNAase-I resistant so long as it remains undisturbed. Whatever this organizational feature is, it must include nearly all the resting chromatin and yet not interfere significantly with the action of micrococcal nuclease or the Ca-Mg endonuclease. If we assume that the hindrance that the superstructure offers to DNAase-I is simply due to the spacing arrangement of the nucleosomes, then this suggests that nucleosome spacing is ordered so that each nucleosome protects its neighbours completely from DNAase-I (MW 31,000)[10] but not at all from micrococcal nuclease (MW 16,800)[8]. This suggests a system of packing with some extremely uniform features if it does not hinder diameters of approximately 34 A^{o} (micrococcal nuclease) but completely hinders diameters of 41 A^{o} (DNAase-I). If this is the case, it is not at all surprising that newly synthesized chromatin is highly DNAase-I susceptible as a progressing zone of DNA synthesis would be expected to cause a transitory upset in chromatin organization and there is cytological evidence of the existence of such upsets[28]. When these results are considered together with the studies of Weintraub[29] on chick chromatin, it seems that the newly-synthesized chromatin may go through at least three stages. The first stage is a very short, DNAase I resistant phase described by Weintraub, the next stage is a longer DNAase-I susceptible phase described in this paper and the final stage is the very highly DNAase-I resistant state of the organized nucleus.

Finally, it should be noted that the powerful destabilizing effect of calcium in the presence of DNAase-I and free Mg^{++} may have more than one explanation. Although this destabilization could be explained in terms of the action of the nuclear Ca-Mg endonuclease, the results of Wiberg[23] suggest that calcium may cause important changes in the catalytic properties of DNAase-I itself. Moreover, there is a serious possibility that calcium stimulated nuclear proteases[30] might also be significant in

this effect.

Calcium destabilization could also be an important factor facilitating the attack of micrococcal nuclease on chromatin although, if this was the case, it would indicate that the calcium destabilization was more likely to be mediated by traces of protease rather than the Ca-Mg endonuclease.

ACKNOWLEDGEMENTS

This work was supported by the Australian Research Grants Committee and the Flinders University Research Budget.

REFERENCES

1. Marshall, A.J. and Burgoyne, L.A. (1976). Nucleic Acid Res. 3, 1101.
2. Burgoyne, L.A. and Mobbs, J. (1975). Nucleic Acid Res. 2, 1551.
3. Hewish, D.R. and Burgoyne, L.A. (1973). Biochem. Biophys. Res. Commun. 52, 504.
4. Burgoyne, L.A., Hewish, D.R. and Mobbs, J. (1974). Biochem. J. 143, 67.
5. Hewish, D.R. and Burgoyne, L.A. (1973). Biochem. Biophys. Res. Commun. 52, 475.
6. Noll, M. (1974). Nature 251, 249.
7. Axel, R. (1975). Biochemistry 14, 2921.
8. Taniuchi, H., Anfinien, C.B. and Sodja, A. (1967). J. Biol. Chem. 242, 4752.
9. Noll, M. (1974). Nucleic Acid Res. 1, 1573.
10. Lindberg, W. (1967). Biochemistry 6, 335.
11. Bradbury, E.M. (1976). Trends in Biochem. Sciences 1, 7.
12. Hyde, J.E. and Walker, I.O. (1975). Nucleic Acid Res. 2, 405.
13. Saffhill, R. and Itzaki, R.F. (1975). Nucleic Acid Res. 2, 113.
14. Varsharsky, A.J., Bakayev, V.V. and Georgiev, G.P. (1976). Nucleic Acid Res. 3, 477.
15. Bradbury, E.M., Molgaard, H.V., Stephens, R.M., Bolund, L.A. and Johns, E.W. (1972). Eur. J. Biochem. 31, 474.
16. Littau, V.C., Burdick, C.J., Allfrey, V.G. and Mirsky, A.E. (1965). Proc. Natl. Acad. Sci. (U.S.A.) 54, 1204.
17. Van Holde, K.E., Sahasrabudde, C.G. and Ramsay-Shaw, B. (1974). Nucleic Acid Res. 1, 1579.
18. Chattergee, S. and Walker, I.O. (1973). Eur. J. Biochem. 34, 519.
19. Simpson, R.T. (1972). Biochemistry 11, 2003.
20. Weintraub, H. and Van Lente, F. (1974). Proc. Natl. Acad. Sci. (U.S.A.) 71, 4249.
21. Sahasrabuddhe, C.G. and Van Holde, K.E. (1974). J. Biol. Chem. 249, 152.
22. Oudet, P., Gross-Bellard, M. and Chambon, P. (1975). Cell 4, 281.
23. Wiberg, J.S. (1958). Arch. Biochem. Biophys. 73, 337.
24. Burton, K. (1956). Biochem. J. 62, 315.
25. Burgoyne, L.A., Waqar, M.A. and Atkinson, M.R. (1970). Biochem. Biophys. Res. Commun. 39, 254.
26. Waqar, M.A., Burgoyne, L.A. and Atkinson, M.R. (1971). Biochem. J. 121, 803, and Waqar, M.A. (unpublished results).
27. Wallace, P.G., Hewish, D.R., Venning, M.M. and Burgoyne, L.A. (1971).

Biochem. J. 125, 47.
28. Prescott, D.M. (1966). J. Cell Biol. 31, 1.
29. Weintraub, H. (1973). Cold Spring Harb. Symp. Quant. Biol. 38, 247.
30. Chong, M.T., Garrard, W.T. and Bonner, J. (1974). Biochemistry 13, 5128.
31. EGTA: ethylene glycol bis(2 aminoethylether)-N-N' tetra acetic acid.
32. Seale, R.L. (1975). Nature 255, 247.