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Nuclease digestion in between and within nucleosomes

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ABSTRACT

In the course of digestions of rat liver nuclei with micrococcal nuclease the size of the nucleosomal DNA is shortened by 50-60 nucleotide pairs from starting lengths of about 200, 400, 600, 800 etc. nucleotide pairs in the monomeric and oligomeric nucleosomes, respectively. Acid soluble DNA material is created relatively slowly as compared to the rate of formation of subnucleosomal material. More DNA with lengths in between the 200, 400 etc. nucleotide pairs of nucleosomal DNA is formed when digestions with micrococcal nuclease are carried out at 0 to 10°C compared to 40°C. With DNAase II, on the other hand, formation of a 200 nucleotide pair pattern instead of the otherwise observed 100 nucleotide pair pattern is favoured at the low temperatures. Apparently, the accessibility of potential cleavage sites in between and within nucleosomes depends strongly on the conditions of digestion. Possible reasons for this dependence are discussed.

INTRODUCTION

It is now well established that chromatin consists of subunits (1-6) which are called ν-bodies (2) or nucleosomes (7). Digestions of nuclei or chromatin with nonspecific nucleases (1,3-5,8-10) and restriction nucleases (11) have contributed much to the current picture of chromatin structure. Several features of the nuclease digestions of chromatin are still unclear, however, which is not surprising if one considers the complexity of nuclease digestions of nucleoprotein in general and the problems of the multicomponent system chromatin in particular. The present study of the time and temperature dependence of digestion may help to clarify some of the points. In addition to micrococcal nuclease, DNAase II was used which had been found recently in our laboratory (12,13) to introduce under certain conditions double strand cuts into the DNA of chromatin at regular distances of 100

instead of 200 nucleotide pairs observed with other nucleases.

METHODS

Isolation of nuclei. Rat liver nuclei were prepared essentially according to the method of Hewish and Burgoyne (1). During tissue homogenization buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris, pH 7.4) did not contain $EGTA^+$ and EDTA. After pelleting for 1 h at 100 000 x g through a gradient of 1.37-2.2 M sucrose, the nuclei were resuspended in buffer A, containing 0.2 mM EGTA and 0.2 mM EDTA and washed at least three times in the same buffer. The washed nuclei were pelleted at 5 000 x g for 2 min and the pellets immediately frozen in liquid nitrogen and stored at -80°C. No changes were observed in nuclease digestion patterns during four months of storage under these conditions.

Digestion of nuclei with micrococcal nuclease. Nuclei were suspended at 0°C in buffer A containing 0.2 mM EDTA, 0.2 mM EGTA and pelleted. The pellet was resuspended in the same buffer to give a final concentration of 0.4-0.5 mg per ml nuclear DNA. 1.6 mM $CaCl_2$ was added and the reaction mixture allowed to stand for a few minutes at the temperature indicated in the text. The reaction was started by the addition of micrococcal nuclease (E.C. 3.1.4.7, Worthington Biochemical Corp., Freehold, New Jersey). Aliquots were taken at different times and the reaction terminated by the addition of EGTA to a final concentration of 10 mM. After addition of SDS (final concentration 0.4%) and 100 µg/ml proteinase K (E. Merck, Darmstadt) the mixtures were incubated for 3 h at 37°C or overnight at 25°C. After extraction with phenol the DNA was precipitated with 2 volumes of ethanol and dissolved for gel electrophoresis in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6.

For determination of the acid soluble DNA material, nuclease digestions were stopped by the addition of 1 vol of 0.8 M perchloric acid and centrifuged. The acid soluble material was determined directly from the supernatant by the method of Martin et al. (14) which is claimed to be specific for desoxyribose.

Digestion of nuclei with DNAase II. Nuclei were washed twice in 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.0, and pelleted. The pellets were suspended in 10 mM Tris-HCl, pH 7.0, 0.1 mM EDTA to a final concentration of 0.4-0.5 mg DNA per ml. Incubations were carried out with 500 units/ml DNAase II (E.C. 3.1.4.6, Worthington Biochemical Corp., Freehold, New Jersey). The reaction was terminated by the addition of 0.4% SDS, 50 mM Tris-HCl, 17 mM EDTA, pH 8.5 (final concentrations) and incubated overnight at 25°C with 100 µg/ml proteinase K. The DNA was isolated for gel electrophoresis as described for micrococcal nuclease digestions.

Gel electrophoresis. Analytical gel electrophoresis of DNA samples was carried out in vertical 1.5-2% agarose slab gels (Sea Kem, Marine Colloids, Rockland, Maine 04841) using the Tris-borate buffer system (15). 1 µg/ml ethidium bromide was present in the gel and the electrophoresis buffer; in some experiments without dye in the electrophoresis buffer, staining was performed afterwards with 1 µg/ml ethidium bromide for 1 h. DNA was visualized in the gels under ultraviolet light, using a UV transilluminator (Ultra Violet Products, Inc., California), and photographed under conditions, where the darkness on the negative film is linearly proportional to the DNA concentration in the gel (11). The negatives were scanned and the peak areas quantitated.

For isolation of DNA from preparative agarose gels electrophoreses were run in 0.6x18 cm tubes. The bands were cut out and DNA was isolated by electrophoretic elution. Ethidium bromide was removed by phenol extraction and ethanol precipitation of the DNA.

The B. subtilis restriction nuclease digest of λdv-1 DNA (16) used for chain length calibration was a gift of M. Steinmetz of our laboratory.

RESULTS AND DISCUSSION

Kinetics of micrococcal nuclease digestion of rat liver nuclei. There is some uncertainty in the literature with respect to the length of the DNA in the nucleosomes (3,5,10, 17-19) and also on the extent of shortening of the DNA by extended nuclease digestion (10,17,19-22). Since it was

necessary for our work to have consistent size determinations we decided to reinvestigate the question. Two issues appeared to be essential in this type of study, the use of sequenced marker DNA fragments and the refinement of coelectrophoresis techniques.

The chain lengths of the λdv-1 DNA fragments employed in our experiments were determined (R.E. Streeck, to be published) relative to the sequenced restriction nuclease fragment H of SV40 DNA (23 and W. Fiers, personal communication). The length of the fragment is taken to be 269 nucleotide pairs (267 nucleotide pairs plus 4 single stranded nucleotides). The slope of the calibration curve was determined with the help of restriction nuclease digests of such satellite DNAs, which contain fragments of multiple unit length. With respect to the second point, it was observed that high amounts of material in a band influence the mobility of immediately preceding and following bands. Such a situation exists especially early and late in digestion when high molecular weight material and DNA from monomeric nucleosomes, respectively, constitute the main part of the load. Here reelectrophoresis of eluted DNA with admixed marker DNA proved to be of advantage.

In order to facilitate comparison with the literature, our data are plotted as in (19). It should be noted, however, that our actual numbers (Fig.1) differ from those of (19). The extrapolated zero time values agree with a figure of 190-200 nucleotide pairs of DNA protected within the nucleosome. During digestion this DNA is shortened by 50-60 nucleotide pairs. Not much importance is attached to the finding of a smaller value for the monomeric nucleosome (Fig.1) since chain length determinations are least exact with the DNA markers employed. The decrease in the size of the trimeric and tetrameric nucleosomes could not be followed beyond 20 min, since by then the bands begin to disappear. The values for monomeric and dimeric nucleosomes appear to approach a plateau although it should not be excluded that, particularly with higher amounts of nuclease, lower "plateau values" can be reached. The finding that 50-60 nucleotide

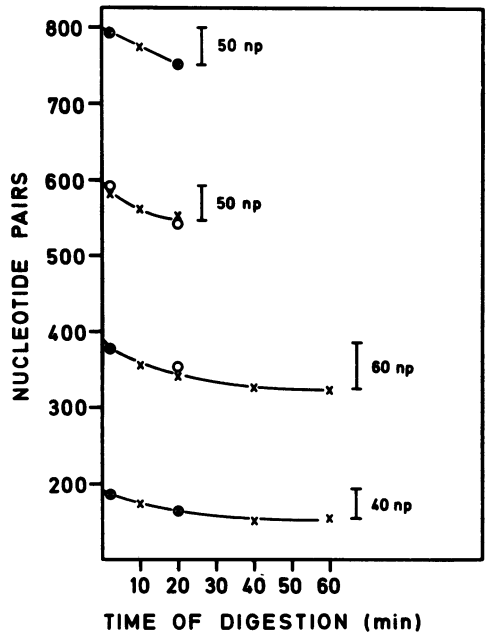

Figure 1. Decrease in chain length of nucleosomal DNA during micrococcal nuclease digestion. Nuclei were digested with 100 units/ml nuclease at 30°C for 2, 10, 20, 40 and 60 min. The digests were electrophoresed on 1.5% agarose slab gels together with DNA markers added to each sample. Values for DNA length (Methods) are plotted (x——x). 2 and 20 min digests were electrophoresed also on a preparative scale; DNA from separated bands was eluted, and reelectrophoresed with added markers (o——o) on 1.5% analytical agarose slab gels (13).

pairs per nucleosome (monomeric nucleosome or termini of an oligomeric one) are more easily digested than the rest of the DNA ties in with current hypotheses on internucleosomal DNA or histone H 1 protected DNA (e.g. 20,21) but does not allow to differentiate between variants of the general picture. This interpretation of Fig. 1 is related to the general observation that bands of nucleosomal DNA are rather broad; we estimated the width to be about 100 nucleotide pairs which agrees well with the above mentioned easily accessible stretch of 50-60 nucleotide pairs per nucleosome.

A quantitative evaluation of the digestion kinetics is presented in Fig. 2. At the time when these experiments were done we hoped to get clues as to the degree of protection of various inter- and intranucleosomal regions from the rate

of appearance of acid soluble DNA material relative to the one of nucleosomal and subnucleosomal material. Some of our results are different from published ones (10,17), but the differences may be explained by the differences in digestion conditions. In general, the kinetic picture is rather complex and allows only a few conclusions to be drawn: subnucleosomal DNA is formed nearly as fast as the nucleosomal DNA; the rate of appearance of acid soluble material correlates well with the breakdown of subnucleosomal material and continued degradation of the terminal sequences of monomeric and oligomeric nucleosomes.

Influence of temperature on the digestion of nuclei with micrococcal nuclease and DNAase II. It has been repeatedly noticed in our work and also by others (e.g.10,17) that not only the bands of nucleosomal DNA have a relatively large width but also that there is some background material between the bands. At least between the monomer and the dimer band this cannot be due to poor resolution of the gels. In the context of this study on nuclease digestions in between and within nucleosomes we were interested in the effect of incubation temperature on the appearance of the"background DNA". At higher temperatures the background between the bands was comparatively low from the very beginning. At low temperatures, however, the background was high in the beginning and stayed rather high until the end of digestion. The observation was confirmed with several different nuclear preparations. The patterns obtained at 40 and 10°C (Fig.3) can be directly compared since the digestions were carried to a similar overall size distribution of the DNA. A semiquantitative evaluation indicates that, going from the higher to the lower temperature, the amount of "background DNA" is at least doubled relative to the amount of DNA in the bands themselves. A digestion experiment at 0°C is included in Fig. 3 in order to show the appearance of a substructure between the monomer and the dimer band, comparable to the subnucleosomal material. Apparently DNA fragments of about 260 and 320 nucleotide pairs length are formed with some preference under these conditions.

In discussing the temperature effect, we would like to

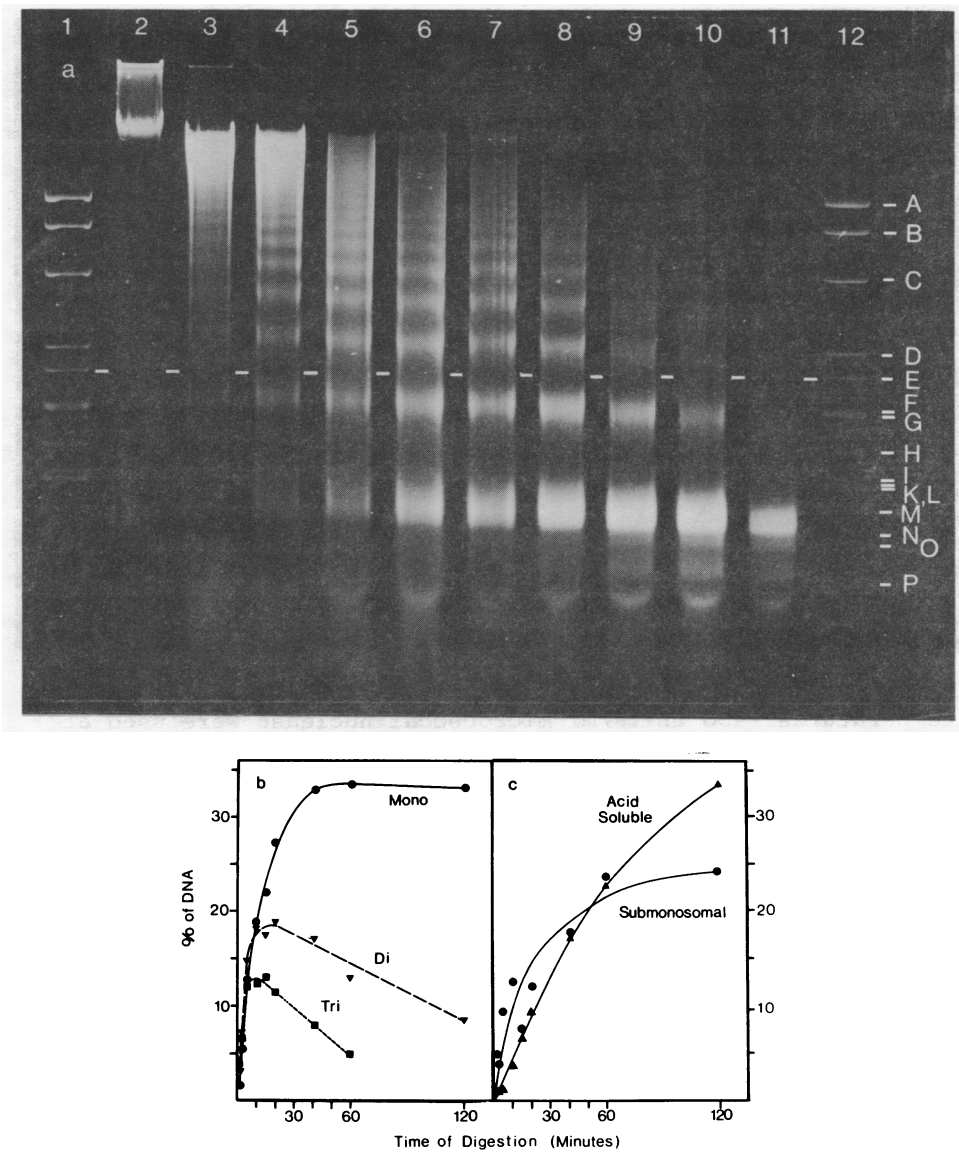

Figure 2. Digestion of DNA in rat liver nuclei for different times with micrococcal nuclease. For the digestion 80 units/ml nuclease was used at 25°C. a. Gel electrophoresis was on 1.5% agarose slab gels, slots 1 and 12 contain DNA standards, slots 2-11 were loaded with nuclear DNA, digested for 0, 1, 2.5, 5, 10, 15, 20, 41, 60 and 120 min, respectively. b. Increase of amounts of nucleosomal DNA during micrococcal nuclease digestion of nuclei, estimated after quantification of the peaks in the scans of the gel shown in a. c. Increase of acid soluble and "submonosomal" DNA material during digestion time.

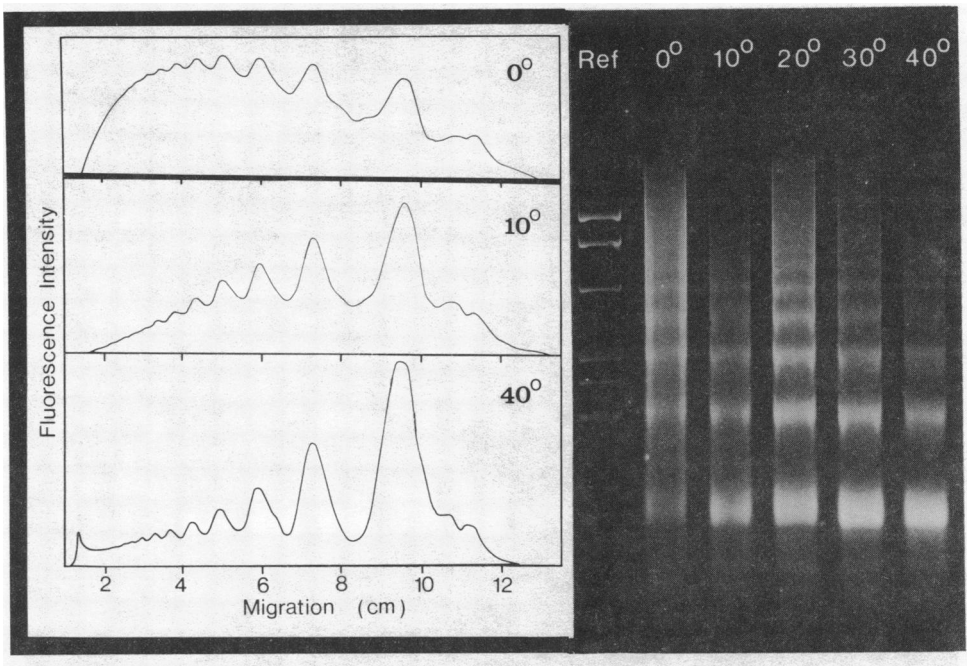

Figure 3. Digestion of DNA in rat liver nuclei at different temperatures. 100 units/ml micrococcal nuclease were used at 40°C for 5 min, at 30°C for 10 min, at 20°C for 20 min, at 10°C for 80 min and at 0°C for 320 min. Electrophoresis was done on 1.5% agarose slab gels. Scans are shown for the 0, 10 and the 40°C digests. λdv-1 DNA fragments were applied as references.

mention four aspects: 1. the mechanism of micrococcal nuclease action itself may change with temperature, for instance with respect to its exonuclease/endonuclease characteristics (24); 2. the affinity of the nuclease to the DNA on one hand and of some protecting proteins, on the other hand, may depend on the temperature in a different way; 3. the conformation of chromatin in the nucleus may depend on the temperature; 4. a heterogeneity of the structure and arrangement of the nucleosomes may contribute to the picture. The observed changes of the digestion patterns are probably not due to only one of the aspects but rather to a combination of several of them. Clearly the overall rate of chromatin digestion is much lower at the low temperatures but a relative increase is observed in the accessibility of sites within the nucleosomes.

After our experiments with micrococcal nuclease it seemed particularly interesting to investigate the temperature dependence of the action of DNAase II. This enzyme had been reported to produce from calf thymus chromatin 11 S particles (18,25) which are characteristic of the nucleosomal arrangement. On the other hand, under certain ionic conditions this enzyme cuts DNA in chromatin at a regular distance of about 100 nucleotide pairs (12,13). It turned out that at 0°C and 10°C formation of the 200 nucleotide pair pattern was strongly favoured while at 37°C only the 100 nucleotide pair pattern was observed (Fig.4). At intermediate temperatures the first type of pattern was formed at the beginning, while the second type of pattern appeared later (Fig.4). When digestions of nuclei were carried out in the presence of

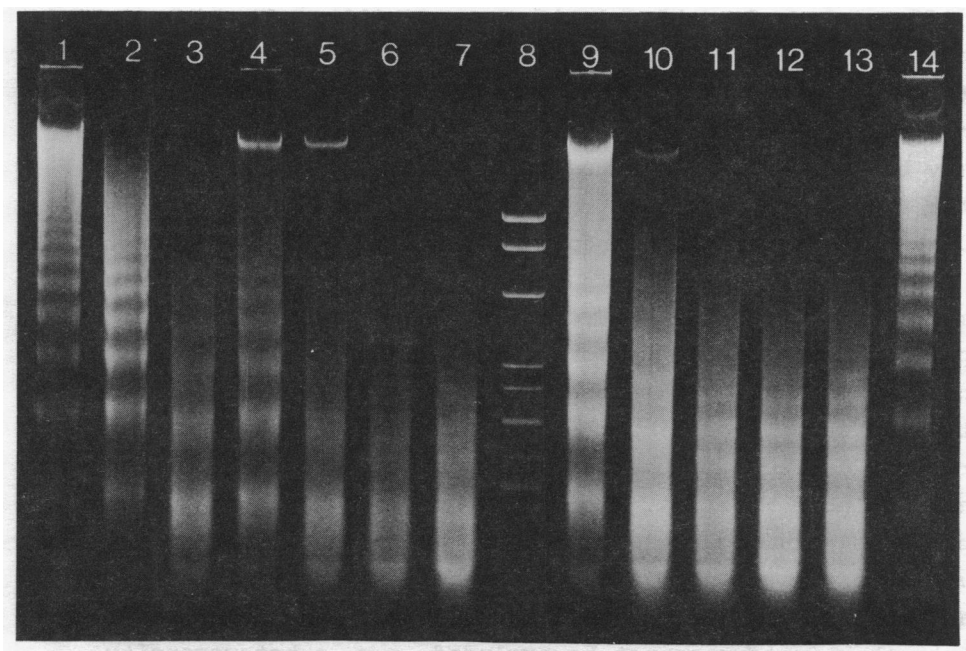

Figure 4. Temperature and time dependence of DNAase II digestion of rat liver nuclei. Washing of nuclei and digestion were carried out as described in Methods. Electrophoresis was on an 1.5% agarose gel. Slots 1 and 14 contain micrococcal nuclease digests of rat liver nuclei as references, slot 8 λdv-1 fragments. DNAase II digestions were done for 5 h at 0°C, slot 2; for 3 h at 10°C, slot 3; for 10, 30, 60 and 90 min at 20°C, slots 4-7, respectively; for 5, 20, 45 and 60 min at 30°C, slots 9-12, respectively; and for 15 min at 37°C, slot 13.

2 mM $CaCl_2$, the 100 nucleotide pair pattern repeat was formed from the very beginning at the low as well as at the higher temperatures. Thus, $CaCl_2$ suppresses the temperature induced changes in the accessibility of DNA. Further details on the role of divalent cations in DNAase II digestions are discussed in (12). It cannot be decided from the cleavage patterns whether 100, 300, 500 etc. nucleotide pair bands are formed from 200, 400, 600 etc. nucleotide pair material or from higher molecular weight chromatin.

The interpretation of the results follows similar lines as in the experiment with micrococcal nuclease. The reaction is generally much slower at the low temperatures and the four aspects mentioned above are also to be considered here. But somehow the combination of different influences yields a different result: the relative accessibility of a site within the nucleosome is higher at the high temperature while at low temperatures with micrococcal nuclease sites within the nucleosome were cleaved with some preference. This difference may be related not only to the different characteristics of the two enzymes but also to the different degree of condensation of chromatin under the two digestion conditions.

Concluding remarks. In the digestion of chromatin with micrococcal nuclease a stretch of DNA of 50-60 nucleotide pairs per nucleosome is more accessible than the 140-150 nucleotide pairs within the core of the nucleosome. During the course of digestion the 50-60 nucleotide pair region is rapidly degraded to smaller products which contribute to the acid soluble material. The differences between the digestion patterns obtained at 10°C and 40°C are probably caused by various factors, for instance changes in the relative accessibility of potential cleavage sites with temperature. But other factors cannot be excluded, e.g. a heterogeneity in the structure and the arrangement of the nucleosomes may contribute to the observed picture. The results of the DNAase II digestion experiments between 0°C and 37°C point to the involvement of conformational changes of the chromatin with temperature. The present results confirm the general view of the nucleosome structure of chromatin and specify

certain aspects of the picture in more detail.

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REFERENCES

1. Hewish, D.R. and Burgoyne, L.A. (1973) Biochem. Biophys. Res. Commun. 52, 504-510.
2. Olins, A.L. and Olins, D.E. (1974) Science 183, 330-332.
3. van Holde, K.E., Sahasrabuddhe, C.G., Shaw, B.R., van Bruggen, E.F.J. and Arnberg, A.C. (1974) Biochem. Biophys. Res. Commun. 60, 1365-1370.
4. Kornberg, R.D. (1974) Science 184, 868-871.
5. Noll, M. (1974) Nature 251, 249-251.
6. Baldwin, J.P., Boseley, P.G., Bradbury, E.M. and Ibel, K. (1975) Nature 253, 245-249.
7. Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Cell 4, 281-300.
8. Bakayev, V.V., Melnickov, A.A., Osicka, V.D. and Varshavsky, A.J. (1975) Nucl. Acids Res. 2, 1401-1419.
9. Noll, M. (1974) Nucl. Acids Res. 1, 1573-1578.
10. Sollner-Webb, B. and Felsenfeld, G. (1975) Biochemistry 14, 2915-2920.
11. Pfeiffer, W., Hörz, W., Igo-Kemenes, T. and Zachau, H.G. (1975) Nature 258, 450-452.
12. Altenburger, W., Hörz, W. and Zachau, H.G. (1976) submitted for publication.
13. Zachau, H.G., Altenburger, W., Greil, W., Hörz, W. and Igo-Kemenes, T. (1976) International Symposium on the Organization and Expression of Eukaryotic Genome, Tehran, Bradbury, E.M. and Javaherian, K. eds., Acad. Press, New York, in press.
14. Martin, R.F., Donohue, D.C. and Finch, L.R. (1972) Anal. Biochem. 47, 562-574.
15. Peacock, A.C. and Dingman, C.W. (1967) Biochemistry 6, 1818-1827.
16. Streeck, R.E. and Hobom, G. (1975) Eur. J. Biochem. 57, 595-606.
17. Axel, R. (1975) Biochemistry 14, 2921-2925.
18. Oosterhof, D.K., Hozier, J.C. and Rill, R.L. (1975) Proc. Natl. Acad. Sci. US. 72, 633-637.
19. Shaw, B.R., Herman, T.M., Kovacic, R.T., Beaudreau, G.S. and van Holde, K.E. (1976) Proc. Natl. Acad. Sci. US. 73, 505-509.
20. Simpson, R.T. and Whitlock, J.P. (1976) Nucl. Acids Res. 3, 117-127.
21. Varshavsky, A.J., Bakayev, V.V. and Georgiev, G.P. (1976) Nucl. Acids Res. 3, 477-492.
22. Keichline, L.D., Villee, C.A. and Wassarman, P.M. (1975) Biochim. Biophys. Acta 425, 84-94.
23. Fiers, W., Rogiers, R., Soeda, E., van de Voorde, A., van Heuverswyn, H., van Herreweghe, J., Volckaert, G. and Yang, R. (1975) Proceedings of the Tenth FEBS Meeting, Chapeville, F. and Grunberg-Mango, M. eds., Vol. 39, p. 17, North Holland/American Elsevier.
24. Laskowski, M. (1961) in Boyer, P.D., Lardy, H. and Myrbäck, K., The Enzymes 5, 142-144, 2nd ed., Acad. Press,

New York, London.
25. Woodcock, C.L.F., Sweetman, H.E. and Frado, L.-L. (1976) Exp. Cell Res. 97, 111-119.

+Abbreviations: EGTA, Ethylene glycol-bis-(2-amino ethyl ether)-N,N'-tetra acetic acid. EDTA, Ethylenediamine tetra acetic acid. SDS, sodium dodecylsulfate.