Probing with DNase I of nucleosomal core particles assembled in vitro in the presence of polyglutamic acid

Jacques D. Retief, B. Trevor Sewell, H. Johann Greyling, Sylva Schwager and Claus von Holt*

Chromatin Research Unit, Council for Scientific and Industrial Research, Department of Biochemistry, University of Cape Town, Private Bag Rondebosch 7700, Cape Town, Republic of South Africa

Received 7 November 1983

DNase I *Polyglutamic acid* *Core particle assembly*

1. INTRODUCTION

Reconstitution of chromatin fibers in vitro from histones and random or unique DNA will become of increasing importance for the execution of structure-function studies on the eukaryotic genome. The reconstitution of nucleosome core particles at low ionic strength in the presence of polyanions introduced in [1] offers potentially great advantages over other methods proposed (review [2]) because in the former method ill-defined tissue extracts or high concentrations of urea and salts are not required to achieve assembly.

The properties of the 145 base pair (bp) DNA-histone octamer particle, assembled in [1], such as the sedimentation coefficient of 11, the ability to introduce supercoils in SV40 DNA and the electron microscopic appearance resemble very closely the natural core particle.

We also regard it as necessary to establish the DNase I susceptibility of a reconstituted particle. Not only would a 10 bp ladder indicate that one face of the DNA is protected by the underlying histone-octamer but an investigation of the kinetic parameters at each of the susceptible sites would be a sensitive probe of the identity or otherwise of the reassembled and natural particles. Polyanion assisted assembly may also lead to the formation of a particle resembling very closely the natural core but consisting only of the histones H3 and H4 as protein component [3]. It is therefore essential to establish whether during polyglutamic acid-assisted assembly such an alternative particle is formed. Should it be octameric with a molecular mass virtually identical to that of the natural octamer it would be indistinguishable from the natural one on cross-linking and SDS gel electrophoresis. Furthermore, the interpretation of structural and functional properties of artificial chromatin fibers depends on whether the polyglutamic acid remains associated with the final product. It would be desirable to assemble cores over shorter periods and not over 16 h under conditions allowing enzymatic degradation of the component DNA and histones by fortuitously contaminating enzymes. This report deals with these aspects.

2. MATERIALS AND METHODS

Chicken erythrocyte nuclei were isolated [4] using the buffer in [5] and briefly digested with micrococcal nuclease (100 units/mg DNA). Soluble chromatin was taken up in 0.25 mM EDTA–Tris at pH 8.0. Long chromatin was isolated from this on 5–20% (w/v) sucrose gradients in 550 mM NaCl, 10 mM Tris–HCl (pH 7.6) and 0.1 mM PMSF as a fraction larger than tetrasomes. The former was collected and concentrated on an Amicon PM 30 membrane. To produce cores this material was redigested in 20 mM

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

sodium acetate, 5 mM Tris–HCl (pH 7.5), 1 mM $CaCl_2$, 2 mM mercaptoethanol and 0.1 mM PMSF. The cores were purified on sucrose gradients as before in the presence of 100 mM NaCl.

DNA was prepared by phenol extractions from appropriate fractions. The preparation of histones via non-denaturing and denaturing methods with the appropriate renaturation procedures and the crystallization of the octamers from these core histone preparations has been described in detail by us [6]. These histone preparations when diluted in the presence of a 2-fold weight excess of polyglutamic acid form instantaneously a clear solution. A stoichiometric mixture of core histones was labelled with ^{125}I at 2 M NaCl, 10 mM Tris–HCl (pH 8.0) in the presence of one IODOBEAD (Pierce) for 20 min leading to complete incorporation of the label. Histones were cross-linked as in [6]. Polyglutamic acid had an M_r of 60000. For labelling purposes 2 mg were treated with 2.5 μCi [^{3}H]acetic anhydride (Amersham) in dioxane [7]. After 30 min at room temperature the sample was taken up in 10 mM NaCl, 10 mM phosphate (pH 8), incubated for a further 30 min at room temperature and fractionated on an Ultra Gel ACA 44 column in 200 mM NaCl to yield polyglutamic acid with spec. act. 0.12–0.15 μCi/mg. Native or assembled cores were labelled at their 5'-ends and subjected to DNase I digestion as in [8]. DNA fragments were fractionated as in [9]. Autoradiography was done on Dupont Cronex X-ray plates. Using the developed plates as matrix, gel slices were made for every 10 bp bands and the radioactivity determined. The kinetics of the reaction were determined for each cutting site as in [8]. Further experimental details are given in the figure legends.

3. RESULTS

The formation of core particles proceeds rapidly and is virtually complete within 30 min (fig.1). The yield of cores if assembled on 145 bp DNA is 76% (not shown). The histone complex in the particle is octameric containing all 4 core histones (fig.2) and is associated with 145 bp of DNA (fig.3). The S value of the particle is identical to that of the natural one. In 121 assembly reactions the centre positions of the two fractions on gradient centrifugation was found to be within 0.25% of the

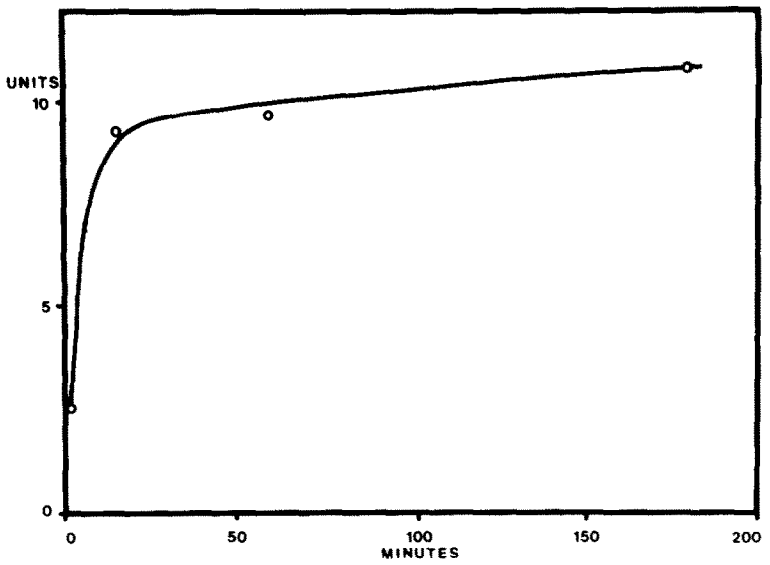


Fig.1. Kinetics of core particle assembly; 800 μg polyglutamic acid, 400 μg core histones, 400 μg DNA in 10 mM Tris–HCl (pH 8), 0.1 mM EDTA, 0.1 nM PMSF, 200 mM NaCl in a volume of 2 ml were incubated at 37°C. The reaction was stopped at the times indicated by the addition of $CaCl_2$ (to give an excess of 1 mM of free calcium) and 1000 units of micrococcal nuclease per mg DNA. The nuclease reaction was terminated after 1 min at 37°C with excess EDTA. The monomeric core particles produced were analysed on a sucrose gradient and the area under the core fraction determined in arbitrary units.

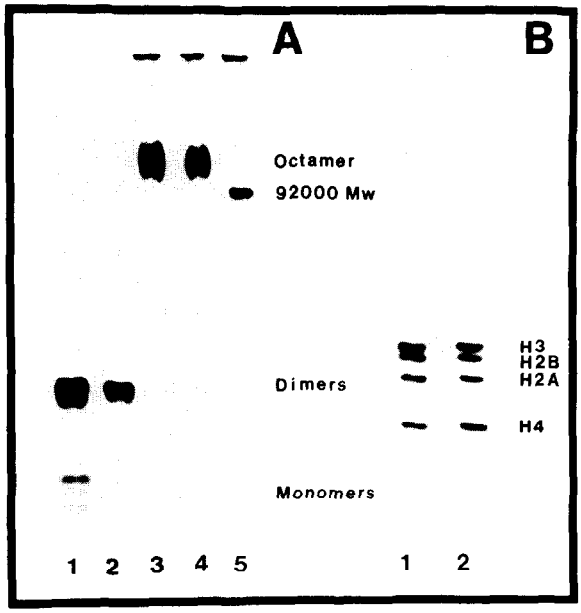


Fig.2. SDS polyacrylamide gel electrophoresis of cross-linked histones. (A) Polyacrylamide gradient of 5–20%, core histones cross-linked in: lane 1, 200 mM NaCl; 2, 500 mM NaCl; 3, 1 M NaCl; 4, 200 mM NaCl plus polyglutamic acid (poly(glu):histone = 2:1); 5, phosphorylase *b* (M_r 92000). (B) 15% polyacrylamide gel. Lane 1, core histones present in the input octamer; 2, core histones isolated from the reconstituted particle. Electrophoresis conditions were as in [12].

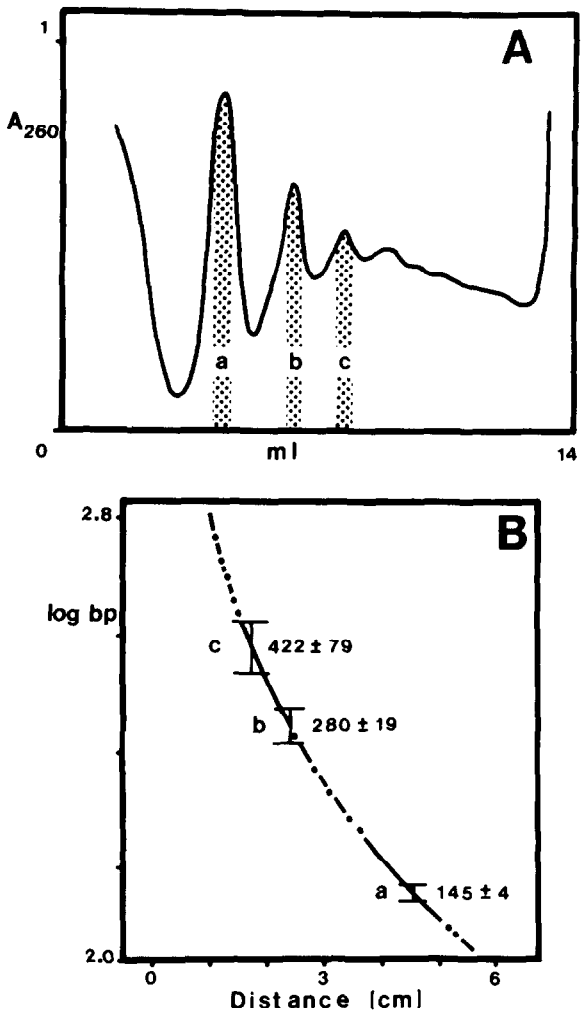


Fig.3. The length of DNA associated with assembled core particles. (A) Cores assembled on long DNA, digested 4 min with 100 units micrococcal nuclease/mg DNA and then fractionated on 5–20% sucrose gradient. (B) Denaturing DNA gel electrophoresis of the DNA from fractions a, b and c. Standards: *Hae*III digest of pBR 322 [13].

elution volume (not shown). Also, the electron microscopic appearance of the fibers and particles (fig.4) resembles closely those reported in [1] and the natural extended primary chromatin fiber as well as the natural core particle.

The polyglutamic acid aiding the assembly is only associated with the octamer in a transient fashion as shown by the displacement of labelled polyglutamic acid by unlabelled polymer from the octamer on exclusion chromatography (fig.5a). Once the octamer is complexed to the DNA to form a chromatin fiber the octamer is stabilised by the DNA only, and on centrifugation leaves the polyglutamic acid at the top of the gradient (fig.5b). The core particles produced through brief micrococcal nuclease digestion from such fibers have been isolated and trimmed with the same enzyme to a very narrow 145 bp population. Such cores were isolated from artificial fibers assembled from a number of histone preparations, undenatured and renatured ones [6]. We had shown previously that all these histone preparations formed octamers with identical conformation as judged by their ability to crystallise [6]. A natural core population isolated from chicken erythrocyte chromatin served as controls. On DNase I digestion the typical distinct 10 bp ladders are indistinguishable from each other (fig.6). For each cutting site the kinetics of the enzyme reaction were established through quantitation of radioactivity of the respective DNA fragment over time.

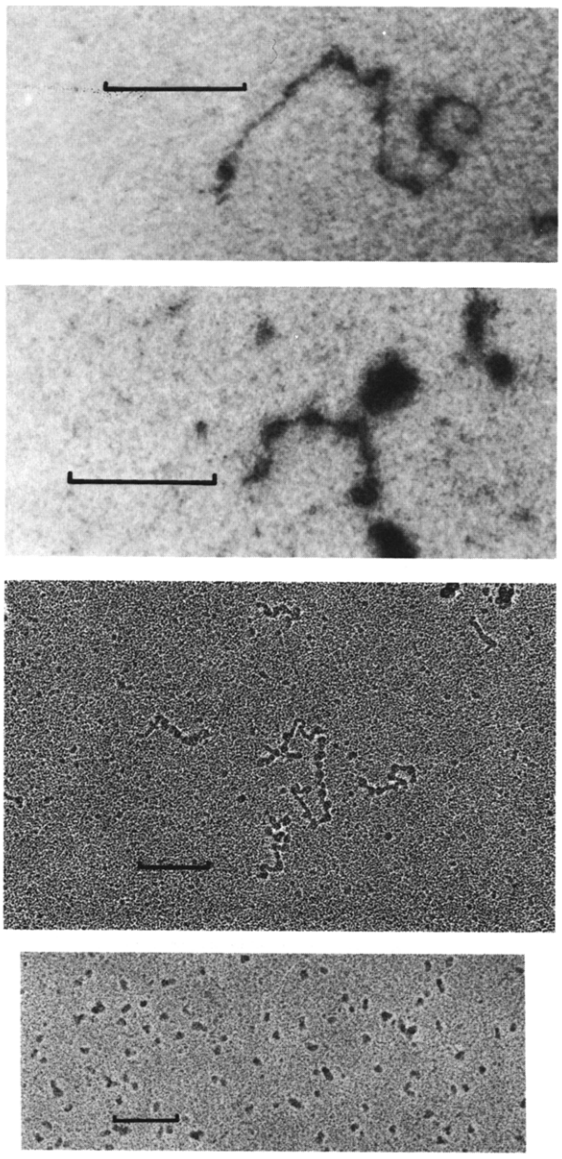


Fig.4. Electron micrographs (from top to bottom: a–d) of poly cores and core particles assembled in the presence of polyglutamic acid. (a,b) Dark field images (printed as negatives). The diameter of the particles is 8 ± 1 nm. (c) Poly cores (shadowed). (d) Core particles (shadowed). The bar in each frame represents 100 nm. Preparations were dialysed for 12 h against 5 mM triethanolamine–HCl (pH 8.0), 1 mM EDTA, diluted 1 in 4 in 0.15% formaldehyde (w/v) in the same buffer and fixed for 1 h at 20°C. BAC was added to a final concentration of 2×10^{-4}%. Dark field: copper grids with approximately 50 Ångstrom carbon films on holey plastic supports were floated for 5 min on a sample drop followed by 1 min on 0.05% (w/v) uranyl acetate, finally the samples on the films were blotted with dry filter paper. Shadowed samples: similar grids but with approximately 200 Ångstrom carbon films were used for sample attachment and uranyl acetate staining as previously, washed with ethanol and blotted as before, followed by rotary shadowing with Pt-C at an angle of 20° in a Balzer coating plant and an electron beam evaporator.

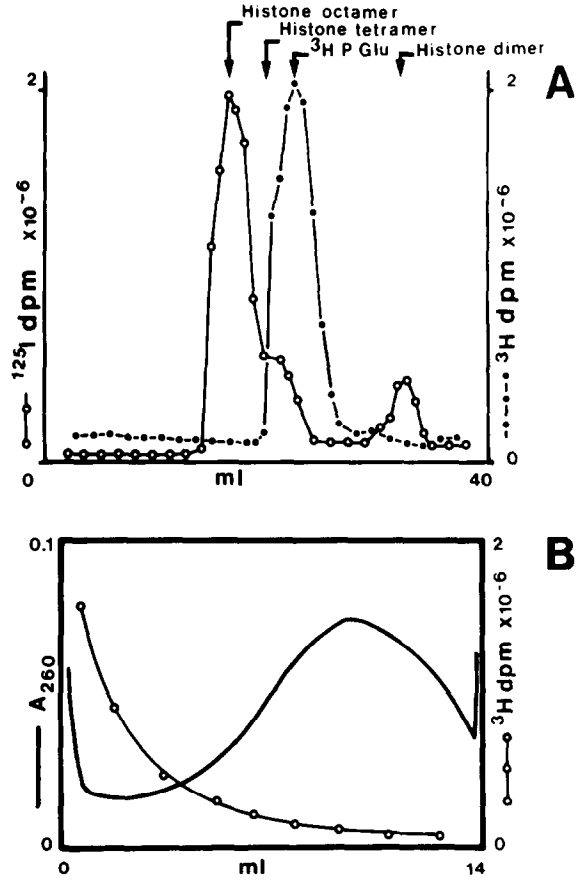


Fig.5. Polyglutamic acid interaction with histone octamers and cores. (A) Core histones were incubated in the presence of polyglutamic acid in 200 mM NaCl, 0.2 mM EDTA, 40 mM Tris–HCl (pH 7.6) at a histone:polyglutamic acid ratio of 1:2. In one experiment labelled core histones and in the other labelled polyglutamic acid were used in the assembly reaction and subsequently the reaction mixture eluted from an ULTRA GEL ACA 44 column (1 × 40 cm) with the same buffer containing 0.1 mg/ml polyglutamic acid. Arrow: octamer evolution volume. (B) 5–20% sucrose gradient centrifugation of nucleosome cores assembled on long DNA in the presence of labelled polyglutamic acid.

Fig.7. Rate constants of DNase I digestion at the various sites on nucleosome cores. The rates displayed are normalised ($\mathfrak{N}$) with respect to the overall cutting rate.

$$\mathfrak{N}_{10n} = \frac{k_{10n}}{\Sigma_{n=1}^{N} k_{10n}}$$

(for a definition of symbols see [6]).

(A) Natural cores. The cutting rate constants, the mean and the standard deviations for each site were determined for each site in 6 different natural core preparations. The bars represent a standard deviation of 34%. (B) Assembled cores. The rate constants were determined as in A for 6 core assembly preparations in which different histone octamer preparations were used; two preparations of sodium chloride extracted histones, two histone octamer preparations eluted from hydroxy apatite and one from renatured acid-extracted histones stored for 2 months in 50% glycerol at −20°C. The preparation of these histone octamers and their crystallisation has been described in detail [6]. The cutting rates at the respective susceptible sites in any of the artificially assembled cores did not differ significantly from preparation to preparation. There are no differences between the artificially assembled cores and the natural cores. All cutting rate constants considered for native and assembled cores have a correlation coefficient greater than 0.860.

→

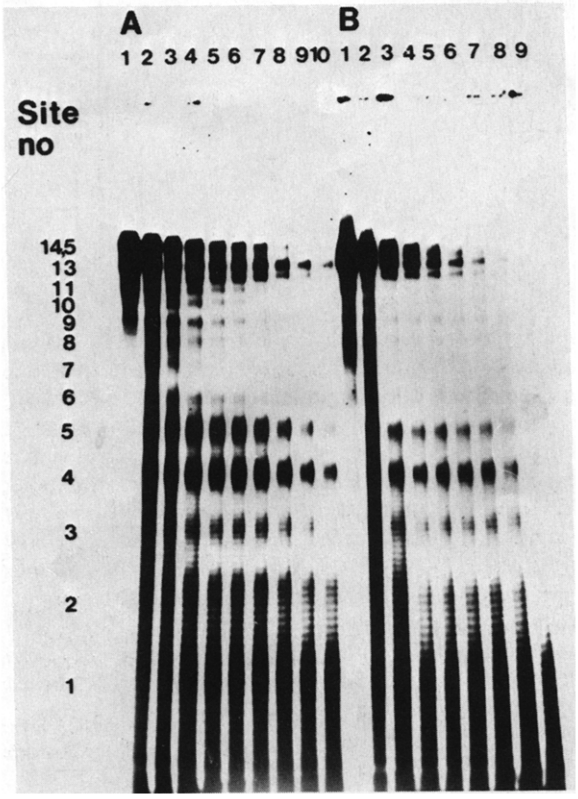
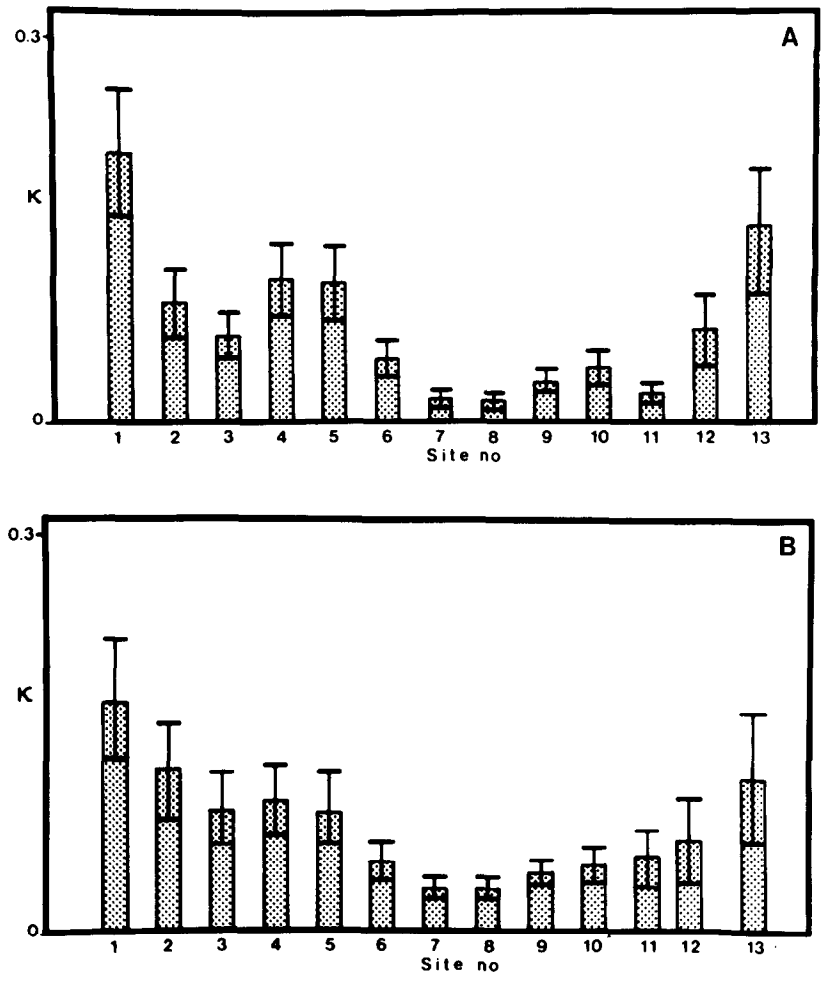


Fig.6. Denaturing DNA gel electrophoresis of DNase I fragments. 5′ ^{32}P-labelled cores were probed with DNase I and the phenol extracted DNA fragments separated electrophoretically. (A) Natural cores, lanes 1–10: digestion for 0, 12, 20, 33, 45, 57, 68, 82, 93 and 104 s. (B) Assembled cores, lanes 1–9: digestion for 0, 9, 21, 33, 45, 55, 67, 78 and 90 s.

This was done for the collection of core particles assembled from the different histone octamers and the natural control particles (fig.7). The kinetics of the DNase I reaction for each site appeared to be typical. All artificially assembled core particles exhibit within the accuracy of the method identical kinetics for the respective typical sites. The DNase I acts on the natural control particles in an identical fashion.

4. DISCUSSION

These investigations have established that core particles are assembled within 30 min and that the association of the core particles with the assembly promoting polyglutamic acid is transient. Under the conditions of assembly only core particles with a complete core histone set are formed, we have found no evidence for a selective assembly of a histone (H3-H4)–DNA complex. The DNase I susceptibilities of core particles assembled from a variety of histone octamers, natural and renatured, are indistinguishable from each other and from those of the natural core particle isolated from chicken erythrocyte chromatin. As in the natural particle one face of the DNA in the artificial particles is protected from DNase I access resulting in the 10 bp ladder [8]. The identical kinetics of the enzyme reaction at the respective sites is interpreted to demonstrate that the steric protection of the DNA at those sites, offered by the histone complex, is identical in both the natural and artificially



assembled particles. We therefore conclude that also the spacial orientation of the histones to each other [10] and with respect to the DNA [11] found in the natural core particles is identical to that assumed by the renatured and natural histone octamers in the artificially assembled nucleosomal cores.

REFERENCES

[1] Stein, A., Whitlock, J.P. jr and Bina, M. (1979) Proc. Natl. Acad. Sci. USA 76, 5000–5004.
[2] Laskey, R.A. and Earnshaw, W.C. (1980) Nature 286, 763–767.
[3] Nelson, T., Wiegard, R. and Brutlag, D. (1981) Biochemistry 20, 2594–2601.
[4] Shaw, B.R., Randall, R.G. and Gross, P.M. (1978) Proceedings of NATO Advanced Study Institute, Erice, Italy. Chromatin Structure and Function (Nicolini, C.A. ed.) pp. 125–136.
[5] Burgoyne, L.A., Hewish, D.R. and Mobbs, J. (1974) Biochem. J. 143, 67–72.
[6] Greyling, H.J., Schwager, S., Sewell, B.T. and Von Holt, C. (1983) Eur. J. Biochem., in press.
[7] Montelaro, R.C. and Rueckert, R.R. (1975) J. Biol. Chem. 250, 1413–1421.
[8] Lutter, L.C. (1978) J. Mol. Biol. 124, 391–420.
[9] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[10] McGhee, T.D. and Felsenfeld, G. (1980) Annu. Rev. Biochem. 49, 1115–1156.
[11] Mirzabekov, A.D., Shick, V.V., Belyavsky, A.V. and Bavykin, S.G. (1978) Proc. Natl. Acad. Sci. USA 75, 4184–4188.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Sutcliffe, J.G. (1978) Nucleic Acids Res. 5, 2721–2728.