EVIDENCE FOR NONHISTONE CHROMOSOMAL PROTEIN KINASE ACTIVITY ASSOCIATED WITH NUCLEOSOMES ISOLATED FROM HeLa S_3 CELLS

I. R. PHILLIPS, E. A. SHEPHARD, W. B. TATCHER, J. L. STEIN[+] and G. S. STEIN

Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL 32610 and [+]*Department of Immunology and Medical Microbiology, University of Florida, Gainesville, FL 32610, USA*

Received 18 June 1979
Revised version received 20 July 1979

1. Introduction

Nonhistone chromosomal proteins and phosphorylation of chromosomal polypeptides have been implicated in the structural and transcriptional properties of the eukaryotic genome [1–6]. However, the relationship between phosphorylated nonhistone chromosomal proteins and the basic structural units of the eukaryotic genome, nucleosomes, remains to date an open-ended question. In an attempt to address the biological significance of nonhistone chromosomal protein phosphorylation, we have examined the representation of phosphorylated nonhistone chromosomal proteins and protein kinase activity in nucleosome cores isolated from exponentially growing HeLa S_3 cells. Evidence is presented which suggests that HeLa cell nucleosome preparations contain protein kinase activity that phosphorylates nonhistone chromosomal proteins but exhibits little ability to phosphorylate core histones. This protein kinase activity decreases with increasing nucleosome oligomer chain lengths; however, nuclease cleavage of oligomers to monosomes results in an increased level of protein kinase activity.

2. Materials and methods

2.1. *Isolation of nucleosome core particles*

Nuclei were prepared essentially as in [7]. All procedures were at 0–4°C unless stated otherwise. Exponentially growing HeLa S_3 cells were washed 3 times in Spinner salts (Grand Island Biol. Co.) and resuspended in homogenisation buffer (0.3 M sucrose–2 mM Mg acetate–10 mM Hepes (pH 7.6)–0.1% Triton X-100) (1 ml/10^7 cells). Cells were allowed to swell for 20 min and were then homogenised by hand for 20 strokes in a tight-fitting glass/glass homogeniser. The homogenate was mixed with an equal volume of heavy sucrose solution (2 M sucrose–5 mM Mg acetate–10 mM Hepes (pH 7.6)) and the mixture was layered over a 15 ml pad of heavy sucrose solution in 38 ml polycarbonate centrifugation tubes. Nuclei were pelleted by centrifugation at 70 000 × *g* for 45 min. Nucleosomes were prepared as in [8]. Nuclear pellets were resuspended in digestion buffer (0.25 M sucrose–15 mM Tris–HCl (pH 7.5)–60 mM KCl–15 mM NaCl–10 mM $MgCl_2$–1 mM $CaCl_2$) to final conc. ~100 A_{260} units/ml. A_{260} was measured after homogenising a sample in 2 M NaCl–5 M urea. Nuclei were pre-incubated at 37°C for 10 min, micrococcal nuclease (6 Sigma units/100 A_{260} units) was added, and the incubation was continued for 20 min. The digestion was stopped by the addition of 1/10th vol. 0.11 M EDTA (pH 7.0). Nuclei were lysed by dialysis for 2–3 h (with vigorous stirring) against 10 mM Tris–HCl (pH 7.5)–1 mM EDTA. To ensure nuclear lysis, the sample was briefly homogenised in a Potter homogeniser after dialysis. The sample was clarified by centrifugation at 1000 × *g* for 5 min. The supernatant was layered over a 5–20% linear sucrose gradient containing 25 mM Tris–HCl (pH 7.5)–0.1 mM EDTA and centrifuged at 25 000 rev./min in a Beckman SW27 rotor for 20 h. Gradients were fractionated using an ISCO density gradient fractionator (Model 640). Fractions from the A_{260} peak at ~11 S

were pooled and dialysed against 10 mM Tris–HCl (pH 7.5)–0.1 mM EDTA. If necessary, nucleosomes were concentrated either by 'reverse dialysis' against dry Sephadex G-50 or with a Collodion Bag apparatus (Schleicher and Schuell, Keene, NH). When all the gradient fractions were to be assayed for protein kinase activity, the resolution of the density gradient centrifugation was increased by centrifuging the nuclear digests on a 5–30% linear sucrose gradient at 36 000 rev./min in a Beckman SW41-Ti rotor for 20 h.

2.2. *Protein kinase assay*

Samples were assayed for protein kinase activity in 50 mM Tris–HCl (pH 8.0)–20 mM $MgCl_2$–3 μM [γ-^{32}P]ATP (3 Ci/mmol). The total volume of the assay mixture was 300 μl. Where indicated, various amounts of exogenous proteins were included. Assays were incubated at 37°C for 30 min unless stated otherwise. The reaction was stopped by the addition of 2 ml 20% trichloroacetic acid–2% sodium pyrophosphate. The precipitate was collected on a Millipore HA 0.45 μm filter and washed with 4 ml 20% trichloroacetic acid–2% sodium pyrophosphate, then with 30 ml 10% trichloroacetic acid. Filters were dissolved in 1 ml Cellosolve (Fisher), 10 ml Cellosolve scintillation cocktail (240 ml Cellosolve–40 ml Liquifluor–720 ml toluene) was added and radioactivity was measured by liquid scintillation spectrometry.

2.3. *Preparation of overdigested chromatin fractions*

Nuclei were prepared and incubated with micrococcal nuclease as above except that 24 Sigma units of enzyme/100 A_{260} units were used and sample was incubated for 60 min. Nuclear digests were centrifuged on 5–30% linear sucrose gradients at 36 000 rev./min for 20 h in a Beckman SW41-Ti rotor.

2.4. *Precipitation of nucleosome cores [9–11]*

Nucleosome core particles (~1 A_{260} units/ml) were made 10 mM with respect to $MgCl_2$ and incubated at room temperature for 10 min. Nucleosomes were pelleted by centrifugation at 15 000 × *g* for 20 min and resuspended in 0.9 ml 2 mM EDTA. Then 0.1 ml 3 M NaCl–50 mM Tris–HCl (pH 8.0) was added.

2.5. *Extraction and electrophoresis of nucleosomal DNA*

Nucleosomal DNA was extracted and analysed electrophoretically as in [8]. Nucleosomes were resuspended in 0.2 M NaCl–10 mM EDTA–50 mM Tris–HCl (pH 8.0)–pancreatic RNase (0.5 μg/100 μg DNA) and incubated at 37°C for 3 h. SDS and pronase were added to final conc. 2% and 3 μg/100 μg DNA, respectively, and the sample was incubated for 4 h at 37°C. The DNA was phenol-extracted, ethanol-precipitated, then dissolved at 1 mg/ml in 4 mM Tris–2 mM sodium acetate–0.2 mM EDTA (pH 7.2)–10% glycerol–0.001% bromophenol blue for electrophoresis on a 10 cm, 3.5% polyacrylamide slab gel at 140 V for 6 h. The gel and electrode compartments contained 40 mM Tris–20 mM sodium acetate–2 mM EDTA (pH 7.2). The gel was pre-electrophoresed at 80 V for several hours. *Hae* III restriction fragments of pBR322 were used as size markers. The gel was stained with ethidium bromide and photographed.

2.6. *Gel electrophoretic analysis of phosphorylated nucleosomal polypeptides*

Nucleosomes were incubated in the same buffer used for kinase assays (see above) except that the specific activity of the [γ-^{32}P]ATP was 30 Ci/mmol. The reaction was stopped by adding 1/10th vol. 20% SDS. The sample was dialysed overnight against 2% SDS–2% 2-mercaptoethanol–10 mM sodium phosphate (pH 7.4)–30% glycerol, boiled for 3 min and electrophoresed [12] at 90 V on a 9 cm, 15% polyacrylamide slab gel containing SDS [8]. There was a 1 cm stacking gel of 6% polyacrylamide. The gel was stained with Coomassie brilliant blue and scanned at 595 nm. The gel was then sliced transversely into 1 mm sections. The gel slices were placed in 5 ml vials, dried and then dissolved at 80°C in 0.2 ml 30% H_2O_2. Triton/toluene scintillation fluid (3 ml) (42 ml Liquifluor–333 ml Triton X-100–625 ml toluene) were added to each vial and radioactivity was determined by liquid scintillation spectrometry.

2.7. *Preparation of nucleosome oligomer particles*

Nucleosome oligomer particles were prepared as in [13–15]. Nuclei were isolated as above. Nuclear pellets were resuspended at 1.5 × 10^8/ml in 0.34 M sucrose–60 mM KCl–15 mM NaCl–0.15 mM spermine–0.5 mM spermidine–15 mM 2-mercaptoethanol–15 mM Tris–HCl (pH 7.4) [16]. Nuclei were incubated at 37°C for 5 min, then $CaCl_2$ was added to final conc. 1 mM. Micrococcal nuclease (3.6 Sigma

units/ml) was added and the nuclei were incubated for a further 2.5 min. The digestion was stopped by adding 0.02 vol. 0.1 M EDTA and chilling on ice. Nuclei were pelleted by centrifugation at 2000 × *g* for 5 min at 4°C and rapidly resuspended at 0°C in 0.2 mM EDTA (pH 7.0) using a Pasteur pipette. Nuclei were lysed during a 5 min incubation at 0°C. The suspension was cleared of nuclear debris by centrifugation at 2000 × *g* for 10 min at 4°C. The supernatant was adjusted to 40 A_{260} units/ml and centrifuged on a 5–30% linear sucrose gradient containing 25 mM Tris–HCl (pH 7.5)–0.1 mM EDTA at 25 000 rev./min (Beckman SW27) for 22 h at 4°C. Gradients were fractionated and A_{260} peaks were pooled, dialysed and concentrated as above.

2.8. *Chemical analyses*

Protein concentration was determined by the method [17]. DNA was determined by A_{260}.

3. Results

3.1. *Identification of protein kinase activity associated with HeLa S_3 cell nucleosomes*

Nucleosome core particles were isolated from HeLa S_3 cells by micrococcal nuclease digestion and sucrose density gradient centrifugation as in section 2. Gel electrophoretic analysis showed that these core particles contained approximately equal amounts of histone H2A, H2B, H3 and H4 together with small amounts of several nonhistone proteins and a 140–150 base pair fragment of DNA. When assayed in vitro, the core particles were found to contain a kinase activity that could phosphorylate endogenous nucleosomal proteins. The kinase activity, as reflected by incorporation of [γ-^{32}P]ATP into acid-precipitable material, increased in a linear manner for ~45 min.

The protein kinase activity may not be a part of the nucleosome core but instead may be part of a non-nucleosomal protein aggregate that has sedimentation properties similar to those of the core particles. To distinguish between these possibilities, the protein kinase activities of sucrose gradient fractions from a HeLa cell nucleosome preparation were compared with those of gradient fractions from HeLa cell nuclei that had been digested with micrococcal nuclease beyond the core particle stage. Analysis of gradient

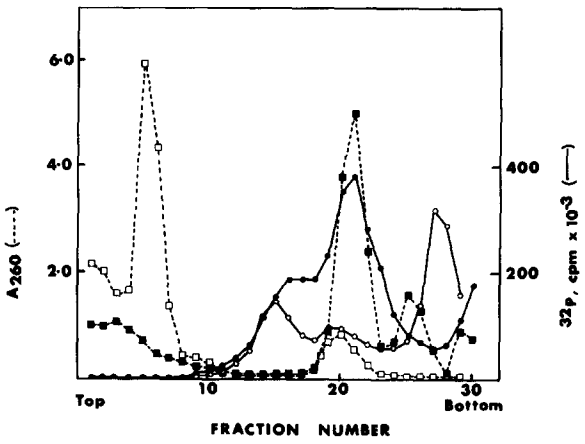


Fig.1. Protein kinase activity associated with nucleosome core particles. Nuclei were treated with micrococcal nuclease (6 Sigma units/100 A_{260} units for 20 min at 37°C) as described in section 2.1. Nuclei were lysed and centrifuged on a 5–30% linear sucrose gradient. A_{260} (■- - -■); protein kinase activity (●——●). Nuclei were treated with micrococcal nuclease (24 Sigma units/100 A_{260} units for 60 min at 37°C) as described in section 2.3, then lysed and centrifuged on a 5–30% linear sucrose gradient. A_{260} (□- - -□); protein kinase activity (○——○). Gradient fractions were assayed for protein kinase activity in the presence of 50 μg casein. [γ-^{32}P]ATP was spec. act. 3 Ci/mmol.

fractions from the nucleosome preparations showed that the major peak of protein kinase activity corresponded with the position of nucleosome core particles (fig.1). When gradient fractions from nuclei digested beyond the core particle stage were assayed, the A_{260} peak corresponding to nucleosomes was greatly reduced with a corresponding increase of A_{260}-absorbing material near the top of the gradient. This decrease in the yield of 11 S nucleosomes coincided with a large decrease in the 11 S protein kinase activity. There was an increase of kinase activity in material recovered near the bottom of the gradient, possibly due to kinase that was released from nucleosomes forming protein aggregates with itself or with other proteins. It therefore appears that breakdown of the nucleosome core particle may lead to release of nucleosome-associated protein kinase activity.

Another approach we pursued to address the problem of whether protein kinase is associated with HeLa cell nucleosomes was to precipitate nucleosomes with 10 mM $MgCl_2$ [9–11]. Although, when nucleo-

Table 1
Association of protein kinase activity with HeLa-cell nucleosomes prior to and following precipitation

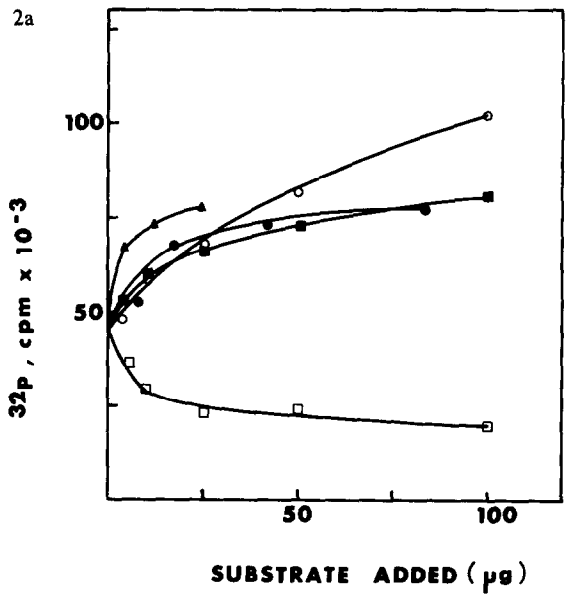
Sample	A_{260}	Protein kinase activity (pmol phosphate incorporated)	
		Endogenous substrate	+ Casein
Core particles before precipitation	1.54	3.62	6.21
Supernatant	0.07	1.52	2.44
Resuspended pellet	0.96	2.35	3.55

Nucleosome core particles were prepared as in section 2.1, and assayed for protein kinase activity as described in section 2.2. Core particles were incubated for 10 min at room temperature in 10 mM $MgCl_2$ and pelleted by centrifugation at 15 000 × g for 20 min. The pellet was resuspended in 0.9 ml 2 mM EDTA, then 0.1 ml 3 M NaCl–50 mM Tris–HCl (pH 8.0) was added. Samples (0.1 ml) of supernatant and resuspended pellets were assayed for DNA content and protein kinase activity

somes were precipitated, some kinase activity remained in the supernatant, ~60% of the kinase was recovered in the pellet (table 1), suggesting that the majority of the protein kinase activity that is detectable in nucleosomes prepared from HeLa cells is indeed associated with the nucleosomes.

3.2. *Substrate preference of nucleosome-associated protein kinase*

The ability of HeLa cell nucleosome-associated protein kinases to utilise various substrates in vitro was evaluated. Nucleosomes were prepared as in section 2 and assayed for protein kinase activity in the presence of various amounts of casein, HeLa-cell nonhistone chromosomal proteins, HeLa-cell nucleosome cores or HeLa-cell histones. In all cases, these substrates were incubated before use at 65°C for 10 min to inactivate endogenous kinase activity. The data in fig.2a indicate that the protein kinase associated with HeLa-cell nucleosomes utilised nucleosomes, nonhistone chromosomal proteins or casein as substrate. Nucleosomes and casein were both phosphorylated to the same extent (~70% above background level) whereas a further enhancement in the level of phosphorylation (~120% above background) was observed when nonhistone chromosomal proteins were assayed. In addition to serving as substrates it is possible that components of the complex and heterogeneous nonhistone chromosomal proteins may activate HeLa cell nucleosome-associated kinase activity. In contrast, histones had a distinct inhibitory effect on HeLa-cell nucleosome-associated protein kinase activity, with a plateau of ~50% inhibition (fig.2a). This inhibition could be due either to a specific effect of histones on the nucleosome-associated enzymes or substrate molecules, or alternatively to a blocking of sites due to non-specific aggregation. Similar inhibitions of chromatin-associated protein kinases by histones



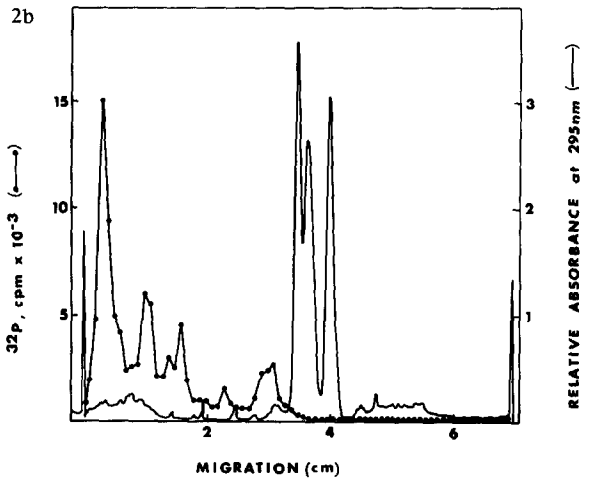


Fig.2. Substrate preference of nucleosome-associated protein kinase. (a) Nucleosome core particles were prepared as described in section 2.1. Nucleosome cores (10 μg DNA) were assayed for protein kinase activity (section 2.2; [γ-^{32}P]ATP was spec. act. 3 Ci/mmol) in the presence of various amounts of nucleosome cores (•); nonhistone chromosomal proteins (○); casein (▪); or histones (□). Kinase activity is plotted with respect to either total nucleosomal proteins (histones and nonhistones) (•) or nucleosomal nonhistone proteins alone (▲). All substrates were incubated before use at 65°C for 10 min to inactivate endogenous kinase activity. The point where the curves meet at the ordinate represents the protein kinase activity of the nucleosome cores towards endogenous substrates. (b) Endogenous nucleosome polypeptides phosphorylated by the nucleosome-associated protien kinase. Nucleosome core particles were prepared as described in section 2,1, incubated with [γ-^{32}P]ATP (spec. act. 3 Ci/mmol) and electrophoresed on an SDS–15% polyacrylamide gel (section 2.6). Scan of Coomassie brilliant blue-stained gel (——); radioactivity profile (•——•).

have been reported [18,19]. Because of the inhibition of nucleosome-associated protein kinase activity by histones, the kinase may phosphorylate the nonhistone components of nucleosomes to a greater extent than is evident when the activity is plotted as a function of total nucleosomal proteins (both histone and nonhistone) (fig.2a).

To establish the complement of endogenous HeLa cell nucleosome polypeptides that are phosphorylated by the HeLa-cell nucleosome-associated kinases in vitro, nucleosomes were incubated in the presence of [γ-^{32}P]ATP and nucleosomal proteins were extracted and analysed by SDS–polyacrylamide gel electrophoresis as in section 2. Although the Coomassie blue-stained electrophoretic profile showed that histones constitute >95% of the nucleosomal proteins, radioactive phosphate was incorporated primarily (>99%) into nonhistone polypeptides of higher molecular weight than the four core histones (fig.2b). Four or five distinct molecular weight classes of nonhistone chromosomal polypeptides were phosphorylated.

3.3. *Distribution of protein kinase activity in nucleosomal oligomers*

The protein kinase activity in nucleosome oligomers and in core particles was compared. Nucleosome monomers, dimers, trimers and tetramers were prepared as in section 2. Although endogenous protein kinase activity was present in all the nucleosome samples, this activity decreased as the nucleosome oligomer chain length increased (table 2), until in tetramers it was only 15% of that found in monomers. A possible explanation of the enhanced level of protein kinase activity in nucleosome monomers is that the kinase activity is not associated with nucleosomes but is instead due to a non-nucleosomal protein aggregate that sediments at the same rate as nucleosome monomers. However, when the oligomer particles were combined and digested to core particles the kinase activity was restored to the monomer level (table 2), suggesting that the latter reservation is not a viable interpretation of our results. We feel that our data are consistent with protein kinase activity being present in nucleosome oligomers but that the increased protein content of the oligomers and/or interactions between nucleosomes results in blocking of the active sites of the enzyme(s) or of the phosphorylation sites of the substrates. These blocking proteins may be released when oligomers are digested to core particles. It should be noted that the degree of inhibition of the kinase activity towards endogenous substrates was greater than that of the enzyme towards exogenous casein (table 2), suggesting that both enzyme and substrate sites may be blocked.

4. Discussion

Our data suggest that HeLa nucleosome preparations contain protein kinase activity. Evidence is presented which is consistent with this kinase activity not being due to contamination of the nucleosome

Table 2
Distribution of protein kinase activity in nucleosome oligomers

Particle	Protein/DNA ratio	Protein kinase activity (pmol phosphate incorporated/ 5 μg DNA)	
		Endogenous substrate	+ Casein
Monomer	1.56	3.30	8.91
Dimer	1.77	0.98	3.27
Trimer	1.87	0.54	2.97
Tetramer	2.00	0.50	2.36
Core (from oligomers)	1.97	3.86	9.67

Nucleosome oligomer particles were prepared as described in section 2.7 and assayed for protein kinase activity (section 2.2) in the presence or absence of casein. Nucleosome core particles were prepared by further digestion of the combined oligomer particles with micrococcal nuclease, and centrifugation through a 5–30% linear sucrose gradient

preparations by a non-nucleosomal protein aggregate. The HeLa-cell nucleosome-associated kinase phosphorylates nonhistone chromosomal proteins but not any of the core histones, and in fact, appears to be inhibited by the addition of histones.

The presence of nucleosome-associated protein kinase activity in HeLa cells is in agreement with observations [20,21] of protein kinase activity associated with nucleosomes isolated from HeLa cells and rat liver, respectively. However, in our hands kinases associated with HeLa-cell nucleosomes phosphorylate nonhistone chromosomal proteins but not histones, whereas significant levels of phosphorylation of H3 histone by HeLa-cell nucleosome-associated protein kinases were observed [20]. The nucleosome-associated protein kinase activity observed in rat liver [21] phosphorylates only nonhistone chromosomal proteins. In contrast, protein kinase activity in nucleosomes isolated from Ehrlich ascites cells was not observed [11]. While these observations appear to be somewhat conflicting, it would not be surprising if apparent differences in results from these laboratories may be attributable to variations in nuclease and/or protease activities in the tissues examined. Subtleties in the procedures employed for preparation of nucleosomes and assay of kinase activities should also be considered.

The limited amount of nonhistone chromosomal proteins present in our HeLa-cell nucleosome preparations suggests that the protein kinase activity may not be associated with all nucleosomes. Thus the possibility arises that protein kinases may be associated only with those nucleosomes in specific regions of chromatin – perhaps those in an 'active' configuration or those containing actively transcribed genetic sequences. Within this context we feel that because the protein kinase activity is decreased with increasing nucleosome chain length, the possibility must be considered that in intact cells the nucleosome-associated protein kinase activity may be less than that observed in isolated nucleosome core particles and that the enzyme(s) may phosphorylate proteins only when nucleosomes are in a more 'open' configuration such as may be found in 'active' regions of the genome. However it should be emphasised that the ability of nucleosome-associated protein kinases to phosphorylate chromosomal proteins in vitro may not reflect the situation present in intact cells.

Acknowledgements

These studies were supported by Grant PCM77-15947 from the National Science Foundation.

References

[1] Stein, G. S., Spelsberg, T. C. and Kleinsmith, L. J. (1974) Science 183, 817–824.
[2] Elgin, S. C. R. and Weintraub, H. (1975) Ann. Rev. Biochem. 44, 725–774.
[3] Busch, H. ed (1978–1979) The Cell Nucleus, vol. 4–7, Academic Press, New York.
[4] Kleinsmith, L. J. (1975) J. Cell. Physiol. 85, 459–475.
[5] Stein, G. S., Stein, J. L. and Thomson, J. A. (1978) Cancer Res. 38, 1181–1201.
[6] Phillips, I. R., Shephard, E. A., Stein, J. L. and Stein, G. S. in: Eukaryotic Gene Regulation (Kolodny, G. ed) CRC Press, West Palm Beach, FL, in press.
[7] Sarma, M. H., Feman, E. R. and Baglioni, C. (1976) Biochim. Biophys. Acta 418, 29–38.
[8] Rill, R. L., Shaw, B. R. and Van Holde, K. E. (1978) in: Methods in Cell Biology (Stein, G. S. et al. eds) vol. 18, pp. 69–103, Academic Press, New York.
[9] Honda, B. M., Baillie, D. L. and Candido, E. P. M. (1974) FEBS Lett. 48, 156–159.
[10] Honda, B. M., Baillie, D. L. and Candido, E. P. M. (1975) J. Biol. Chem. 250, 4643–4647.
[11] Böhm, J., Keil, G. and Knippers, R. (1977) Eur. J. Biochem. 78, 251–266.
[12] Laemmli, U. K. (1970) Nature 227, 680–685.
[13] Noll, M., Thomas, J. O. and Kornberg, R. D. (1975) Science 187, 1203–1206.
[14] Finch, J. T., Noll, M. and Kornberg, R. D. (1975) Proc. Natl. Acad. Sci. USA 72, 3320–3322.
[15] Marion, C. and Roux, B. (1978) Nucleic Acids Res. 5, 4431–4449.
[16] Hewish, D. R. and Burgoyne, L. A. (1973) Biochem. Biophys. Res. Commun. 52, 504–510.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Johnson, E. M., Vidali, G., Littau, V. C. and Allfrey, V. G. (1973) J. Biol. Chem. 248, 7595–7600.
[19] Phillips, I. R., Shephard, E. A., Stein, J. L., Kleinsmith, L. J. and Stein, G. S. (1979) submitted.
[20] Simpson, R. T. (1978) Nucleic Acids Res. 5, 1109–1119.
[21] Defer, N., Kitzis, A., Levy, F., Tichonicky, L., Sabatier, M.-M. and Kruh, J. (1978) Eur. J. Biochem. 88, 583–591.