Volume 306, number 1, 66–70 FEBS 11285 July 1992

The lysine-rich H1 histones from the slime moulds, *Physarum polycephalum* and *Dictyostelium discoideum* lack phosphorylation sites recognised by cyclic AMP-dependent protein kinase in vitro

Richard J. Heads[a*], Brian G. Carpenter[a], Howard V. Rickenberg[b,c**] and Timothy C. Chambers[b***]

[a]*Biophysics Section, School of Biological Sciences, Portsmouth Polytechnic, Portsmouth, UK,* [b]*Department of Molecular and Cellular Biology, National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO 80206, USA* and [c]*Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Centre, Denver, CO 80262, USA*

Received 29 May 1992

Calcium chloride-extracted histones were prepared from nuclei of the slime moulds, *Physarum polycephalum* and *Dictyostelium discoideum*, and phosphorylation by purified preparations of cyclic AMP-dependent protein kinase (cAMP-d PK) and growth-associated H1 histone kinase (HKG) examined and compared. Among the major histone fractions and other proteins in the two preparations, the H1 histones from both organisms were found to be effective and exclusive substrates for HKG. cAMP-d PK, which phosphorylates mammalian H1 histone and certain, in particular H2B , of the mammalian core histones, phosphorylated several of the core histones from both slime moulds but did not phosphorylate H1 histone from either. The slime mould H1s remained ineffective substrates for cAMP-d PK even after extensive alkaline phsophatase treatment of the histone preparations. Additional studies demonstrated that the lack of slime mould H1 phosphorylation by cAMP-d PK was not due to competition of the H1 molecules with the core histones for the kinase. Our studies suggest that H1 histones from these organisms, whilst clearly containing sites for phosphorylation by HKG, apparently lack phosphorylation sites recognised by cAMP-d PK. Thus, the mediation of specific nuclear functions by cAMP-dependent phosphorylation of H1 in higher organisms may not occur or be required in these lower eukaryotes.

Histone H1; Phosphorylation; cAMP-dependent; *Physarum polycephalum*; *Dictyostelium discoideum*

1. INTRODUCTION

The lysine-rich H1 histones play a fundamental role in maintaining the organisation of higher order chromatin structure (see [1] and [2] for recent reviews). Two distinct types of H1 phosphorylation occur in vivo. In cells undergoing growth and division, extensive phosphorylation of H1 at multiple sites is catalysed by a cyclic nucleotide-independent protein kinase termed growth-associated H1 histone kinase (HKG) [3]. This enzyme is a homolog of the yeast cdc2+/cdc28 protein kinases which control entry into mitosis [4]. The occurrence of two HKG activities have been demonstrated in *Physarum* and shown to peak prior to maximum phosphorylation of H1 [5]. These kinases have been shown to advance mitosis in *Physarum* [5], thereby supporting the hypothesis that this type of phosphorylation is involved in chromatin condensation at mitosis. Mueller et al. [6] have shown that *Physarum* H1 is hyperphosphorylated at metaphase of the cell cycle, containing up to 24 sites of modification. These sites are located principally in the C-terminal domain, as observed for metaphase-related phosphorylation of mammalian H1s [7,8]. A second type of phosphorylation of H1 occurs in response to elevated levels of cAMP. This involves phosphorylation of a single serine residue, present in the N-terminal domain of most, but not all, mammalian H1 subtypes [9], by cAMP-dependent protein kinase (cAMP-d PK) [10]. Phosphorylation at different sites may promote differential modulation of the interaction of histone H1 with other structural elements of chromatin, leading to altered nuclear structure and function. The core histones are not phosphorylated by HKG but undergo cAMP-d phosphorylation in vitro [11].

The slime moulds, *P. polycephalum* and *D. discoideum*, have proven extremely useful as experimental models for cell cycle regulation and cellular differentiation, respectively. The histones from these organisms have been partially characterised [12,13] but knowledge of the specificity of different protein kinases in the phosphorylation of individual histone components is lacking. We have therefore prepared histones from both slime moulds and examined phosphorylation in vitro by HKG and cAMP-d PK.

Present addresses: *The Hatter Cardiovascular Studies Unit, Division of Cardiology, University College Hospital, Gower Street, London, WC1E6AU, UK. **Immunotech, Luminy, Case 915, 13288 Marseille, Cedex 9, France. ***Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, USA.

Correspondence address: R.J. Heads, The Hatter Cardiovascular Studies Unit, Division of Cardiology, University College Hospital, Gower Street, London, WC1E6AU, UK. Fax: (44) (71) 388 5095.

Published by Elsevier Science Publishers B.V.

2. MATERIALS AND METHODS

2.1. *Materials*

Total histones and H1 histone from calf thymus [14], the heat-stable inhibitor of cAMP-d PK (PKIn) from rabbit skeletal muscle [15] and Novikoff rat hepatoma HKG [16] were prepared as described in the references cited. The purified catalytic subunit of bovine heart cAMP-d PK was a generous gift of Dr. E.G. Krebs, University of Washington, Seattle, WA, USA.

2.2. *Cell culture and histone isolation*

P. polycephalum, strain M_3C VIII, was cultured as microplasmodia in suspension and total histones prepared by $CaCl_2$ extraction as reported previously [17]. *D discoideum*, strain AX3/RC4, was grown axenically and nuclei prepared as previously described [18]; total histones were prepared as above [17].

2.3. *Phosphorylation studies*

Reaction mixtures of 0.1 ml contained 1 $mg \cdot ml^{-1}$ total histones or 0.2 $mg \cdot ml^{-1}$ calf thymus H1, in 25 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol and 0.5 mM γ-[^{32}P]ATP (3000 cpm $mmol^{-1}$), PKIn (1 μg) was added where indicated. Reactions were initiated by addition of either cAMP-d PK of HKG, using an amount sufficient to catalyse the transfer to calf thymus H1 histone of 1 nmol of phosphate per 30 min at 37°C. Reaction mixtures were incubated for 50 min at 37°C and were terminated by the addition of EDTA to 10 mM. Samples were analysed by SDS-PAGE using 15% acrylamide separation gels [19]. Gels were stained with Coomassie blue, destained, dried and exposed, with the aid of intensifying screens, to X-ray film at −70°C for periods of 3–20 h.

2.4. *Dephosphorylation of histones*

Total histones were dissolved in 50 mM Tris-HCl, pH 9.0, 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ at a concentration of 5 $mg \cdot ml^{-1}$ and incubated at 30°C for 24 h with 75 U of calf intestine alkaline phosphatase (Boehringer, Mannheim). Histones were recovered by precipitation with 25% trichloroacetic acid, washed in acidified acetone (0.1 ml conc. HCl per 100 ml acetone), washed in acetone and dried under vacuum. In control experiments using ^{32}P-labelled calf thymus H1 histone phosphorylated by cAMP-d PK in vitro, these conditions resulted in removal of 95% of protein-bound [^{32}P]phosphate (see Fig. 1, panel B).

3. RESULTS

Fig. 1 (panel A) shows analysis by SDS-PAGE of the H1-containing regions of the histone preparations used in this study. Core histones from calf, *Physarum* and *Dictyostelium* have similar mobilities, consistent with the highly conserved nature of these proteins, except that the slime mould H2A's are larger than calf H2A [12] (not shown). However, the H1 histones from these sources have different mobilities. Calf H1 migrates with an apparent M_r of 33,000; *Physarum* H1 is unusually large [12] and has an apparent M_r of 65,000 and *Dictyostelium* H1 has an apparent M_r of 28,000 [13]. These values are considerably higher than the actual molecular weights due to the anomalous migration of histones in SDS-gels [20].

Phosphorylation of calf thymus total histone was first examined to confirm the specificities of the protein kinase and protein kinase inhibitor preparations. Fig. 1 (panel B) shows phosphorylation of calf thymus H1 by bovine cAMP-d PK catalytic subunit (lane 1) and subsequent dephosphorylation by alkaline phosphatase (lane 2). cAMP-d PK phosphorylated H1 and, to different extents, all four of the core histones, with H2B as the prefered substrate (data not shown). HKG phosphorylated only H1 and none of the core histones as reported previously [21], and phosphorylation by cAMP-d PK but not by HKG was inhibited by PKIn (data not shown).

Phosphorylation of *Physarum* total histones is shown in Fig. 2. The exclusive substrate for HKG is H1 (lane 1). In contrast, bovine cAMP-d PK did not phosphorylate H1, but did phosphorylate some of the core histones, notably H4 and a component of the H2B/H3 band (lane 2). Identical results were obtained if the *Physarum* histone preparation was pretreated with alkaline

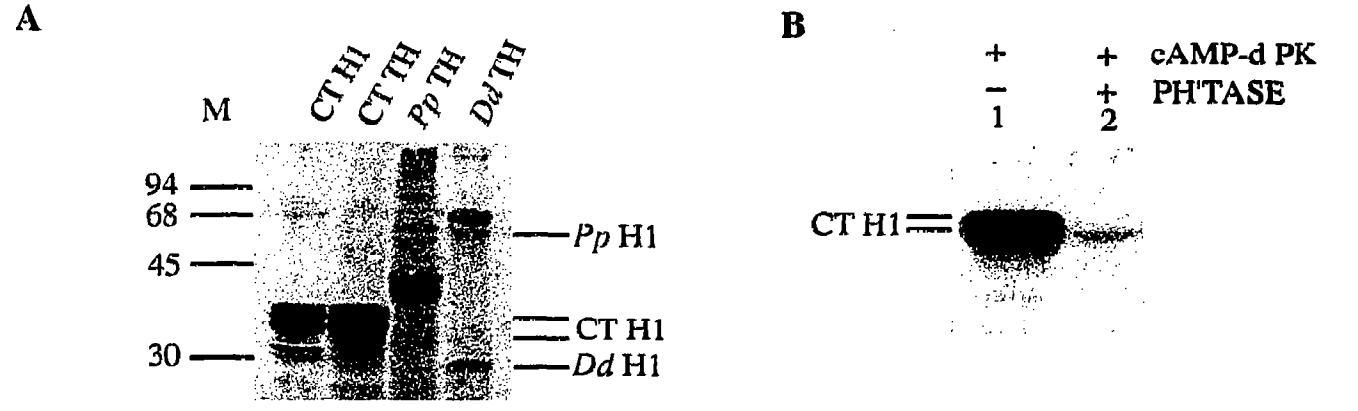

Fig. 1. (Panel A) Analysis of histone preparations used in this study by 15% acrylamide SDS-PAGE and subsequent Coomassie Blue staining. Lane 1, 8 μg calf thymus H1 (CT H1); lane 2, 50 μg calf thymus total histone (CT TH); lane 3, 50 μg *Physarum* total histone (*Pp* TH); lane 4, 50 μg *Dictyostelium* total histones (*Dd* TH). Only the H1-containing regions are shown. M, positions of mol. wt. standards shown in the left. Relative mobilities of the various H1s are shown on the right. H1s were identified by comparison with published data [12,13,17,20] and with purified H1 fractions from the same sources (i.e. see lanes 1 and 2 for CT H1). (Panel B) Phosphorylation of purified calf thymus H1 and subsequent dephosphorylation. Lane 1, phosphorylation of calf thymus H1 by bovine cAMP-d PK catalytic subunit; lane 2, subsequent dephosphorylation by calf intestine alkaline phosphatase (Boehringer).

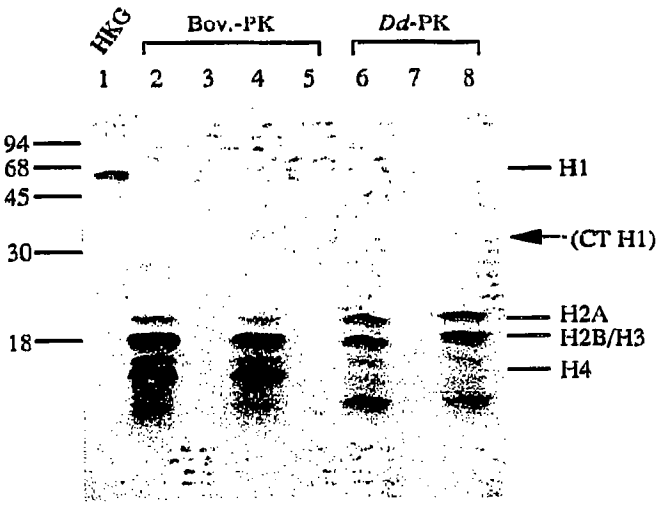

Fig. 2. Phosphorylation of *Physarum* histones. Total histones were phosphorylated as described in Materials and Methods and 50 μg analysed by SDS-PAGE, the gel dried and autoradiographed for 3 h at −70°C. The autoradiograph shows ^{32}P-incorporation into the histones. Lanes 2–5 show phosphorylation by the bovine cAMP-d PK catalytic subunit. Lanes 6–8 show phosphorylation by the *Dictyostelium* catalytic subunit. M, mol. wt. standards; lane 1, HKG treated; lanes 2 and 6, cAMP-d PK treated; lanes 3 and 7, cAMP-d PK+PKIn; lanes 4 and 8, cAMP-d PK+0.1 μg calf thymus H1 (arrow); lane 5, cAMP-d PK+PKIn+0.1 μg calf thymus H1.

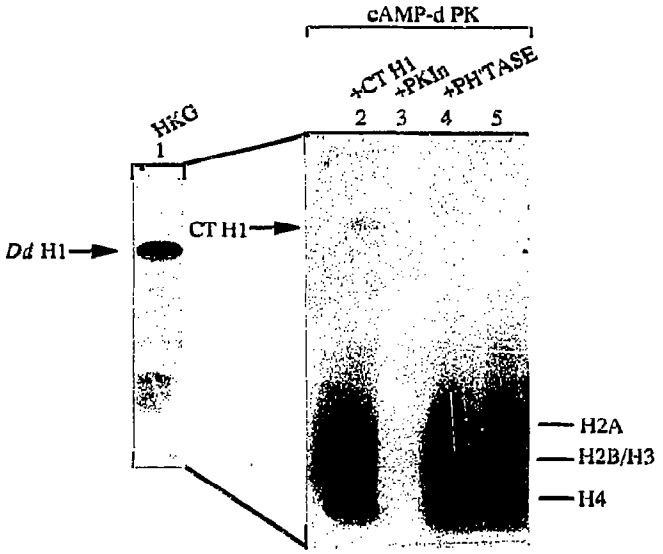

Fig. 3. Phosphorylation of *Distyostelium* histones. Total histones were phosphorylated using the bovine cAMP-d PK catalytic subunit as described in Materials and Methods and 50 μg analysed by SDS-PAGE and autoradiography as shown. Lane 1, HKG treated; lane 2, cAMP-d PK+0.1 μg calf thymus H1 (arrow); lane 3, cAMP-d PK+PKIn; lane 4, cAMP-d PK, histones pretreated with alkaline phosphatase; lane 5, cAMP-d PK treatment alone.

phosphatase (data not shown). Phosphorylation by cAMP-d PK was completely inhibited by PKIn (lane 3). If a trace of calf thymus H1 was added to the reaction mixture, the mammalian H1 was phosphorylated by cAMP-d PK and the pattern of phosphorylation of *Physarum* histones was unchanged (lane 4). The use of this control suggests that the lack of phosphorylation of *Physarum* H1 by cAMP-d PK is not due to a competition effect with the core histones, some of which are effective substrates for this kinase.

This experiment was repeated with partially purified cAMP-d PK from *Dictyostelium*, prepared by a chromatofocussing step, as described previously [22], in place of the bovine cAMP-d PK. Both calf thymus H1 and core histones were phosphorylated by this preparation, i.e. in Fig. 2, lane 8 it can be seen that the trace of calf thymus H1 added is phosphorylated. Neither *Physarum* nor *Dictyostelium* H1 were phosphorylated by the *Dictyostelium* cAMP-d PK, but this kinase preparation did phosphorylate certain of the core histones, notably H2A, H2B and a non-histone protein migrating faster than H4 (Fig. 2). There was a clear difference in substrate specificity between the bovine and *Dictyostelium* kinase fractions amongst the core histones. Whilst the basis of this difference remains to be determined, it is of potential functional significance. Since *Physarum* H1 was not phosphorylated by either enzyme, this must be due to lack of a cAMP-d phosphorylation site rather than a difference in substrate specificity between the two enzymes. Additionally, the lack of slime mould H1 phosphorylation using total histones as a substrate was also observed in preparations enriched in H1 by differential solubility in 0.5 M perchloric acid (data not shown).

Similar studies were conducted with *Dictyostelium* histones (Fig. 3). *Dictyostelium* H1 was the preferred substrate for HKG (lane 1) but was not phosphorylated by bovine cAMP-d PK, although some of the core histones were substrates for cAMP-d PK (lanes 2–5). Phosphorylation of *Dictyostelium* core histones by cAMP-d PK appeared to be more uniform than for the *Physarum* histones (Fig. 2). Alkaline phosphatase pre-treatment did not influence the phosphorylation pattern by cAMP-d PK (lane 4), but phosphorylation was inhibited by PKIn (lane 3). A trace amount of calf thymus H1 when added was phosphorylated (lane 2).

4. DISCUSSION

The findings presented here show that H1 histones from *Physarum polycephalum* and *Dictyostelium discoidium* are substrates for HKG but are not substrates for either heterologous or homologous preparations of cAMP-dependent protein kinases when analysed in vitro. These H1 histones remained ineffective as substrates for cAMP-d PK even after extensive treatment with alkaline phosphatase under conditions which efficiently dephosphorylated ^{32}P-labelled mammalian his-

tone H1. This would appear to rule out the unlikely possibility that the H1s were isolated in a fully (cAMP-dependent) phosphorylated state, thus preventing further phosphorylation in vitro. The lack of slime mould H1 phosphorylation by cAMP-d PK was not due to competition effects, as indicated by 'spiking' the reaction with mammalian H1. In addition, preparations enriched in H1 by perchloric acid extraction were still ineffective as substrates for cAMP-d PK. Loss of a cAMP-d phosphorylation site due to proteolysis during isolation is very unlikely, since the size of the H1 molecules on SDS gels corresponded with those reported for the fully intact H1 molecules described by others [12,13]. The results, therefore, strongly suggest that the slime mould H1's lack a phosphorylation site(s) recognised by cAMP-d PK. This suggestion is supported by earlier work indicating the *Physarum* H1 is not phosphorylated by homologous cAMP-d PK derived from *Physarum* nuclei [23].

Most mammalian H1 subcomponents are phosphorylated in vitro in cAMP-d PK at a single serine residue (Ser-37 in rabbit thymus H1) in the amino terminal region of the molecule [9]. Phosphorylation at this site in vivo of a small proportion of H1 molecules has been observed in a number of cell types following administration of agents which elevate cyclic AMP concentrations [24–26]. Interestingly, certain H1 subcomponents are insensitive to phosphorylation by cAMP-d PK. Subcomponent 3 of rabbit thymus H1, for example, has an alanine in place of serine at position 37 [9]. Our findings suggest the *Physarum* and *Dictyostelium* H1 histones are in a similar class, and it appears that the full complement of H1 histones from these slime moulds lack cAMP-dependent phosphorylation sites.

Since the primary sequences for *Physarum* and *Dictyostelium* H1s are not available, we cannot predict whether the cAMP-d phosphoacceptor sites (i.e.Ser-37 and others) are present but not phosphorylated in vitro under the conditions used in this study. However, Ajiro et al. [27] have shown that the response of five different H1 subtypes (H1a–e) to cAMP-d PK both in vivo and in vitro is subtype-specific in mouse neuroblastoma cells. H1c was phosphorylated at a novel site in a cAMP-dependent manner during differentiation and, whereas H1c, H1d and H1e were phosphorylated at Ser-37 in vitro, H1a and H1b lacked this site, i.e. in H1a Ser-37 is replaced by Thr and H1b did not have a homologous peptide.

A cAMP-d kinase activity from sea urchin eggs (SP kinase) has been shown to specifically phosphorylate the double repeat of the SPKK DNA binding motif Ser-Pro-Arg-Lys-Ser-Pro-Arg-Lys of sperm H1 during fertilisation, decreasing its DNA binding ability [28]. Interestingly, there are 12 potential phosphorylation sites in both the N- and C-terminal domains and therefore cAMP-d phosphorylation involves sites other than Ser-37. Also in this study, SP kinase and bovine A-kinase had different substrate specificities within the N-terminal peptides, as did the *Dictyostelium* and bovine catalytic subunits in our study, at least amongst the core histones. The difference in substrate specificity amongst different H1 subtypes may be reflected in vivo by a differential distribution in chromatin and hence may add a further degree of subtlety to the regulation of chromatin structure during differentiation.

Although cAMP-dependent phosphorylation of mammalian H1 appears to be under hormonal control, a functional role for this modification has yet to be firmly established. Earlier evidence suggested that H1 phosphorylation by cAMP-d PK reduced the ability of the histone to block RNA synthesis on reconstituted chromatin templates [29]. This would infer that phosphorylation of H1 at N-terminal sites reduces its ability to stabilise a transcriptionaly inactive chromatin conformation. This type of phosphorylation may, therefore, enhance the ability of trans-acting factors to displace H1 and gain access to nucleosome-masked regulatory DNA elements (see [30] for review). Since regions of active chromatin do contain nucleosomes and H1, differential modification of core histone domains may cause disruption of the histone octamer through changes in the interaction of these domains with the DNA or other core histones. This would allow destabilisation of nucleosomes with possible displacement and passage of the RNA polymerase [30,31]. On the basis of our results we speculate that any control of gene expression exerted by such phosphorylation in some H1 subcomponents in higher organisms may not occur in these lower eukaryotes. This raises the important question of why these lower eukaryotes may have a different regulatory mechanism with respect to histone H1 modification by phosphorylation.

Acknowledgements: The authors would like to express their gratitude to Dr. T.A. Langan for helpful advice throughout the course of this work.

REFERENCES

[1] Garrard, W.T. (1991) Bioessays 13, 87–88.
[2] Baldwin, J.P. (1992) Curr. Op. Struc. Biol. 2, 78–83.
[3] Matthews, H.R. and Huebner, V.D. (1985) Mol. Cell Biochem. 59, 81–99.
[4] Langan, T.A., Gantier, J., Lohka, M., Hollingworth, R., Moreno, S., Nurse, P., Maller, J. and Sclafani, R.A. (1989) Mol. Cell Biol. 9, 3860–3868.
[5] Inglis, R.J., Langan, T.A., Matthews, H.R., Hardie, D.G. and Bradbury, E.M. (1976) Exp. Cell Res. 97, 418–425.
[6] Mueller, R.D., Yasuda, H., Hatch, C.L., Bonner, W.M. and Bradbury, E.M. (1985) J. Biol. Chem. 260, 5147–5153.
[7] Langan, T.A., Zeilig, C.E. and Leichtling, B. (1980) In: Protein Phosphorylation in Bioregulation, (Thomas, G., Podesta, E.J. and Gordon, J. eds.) pp. 70–82.
[8] Gurley, L.R., D'anna, J.A., Barham, S.S., Deaven, L.L. and Tobey, R.A. (1978) Eur. J. Biochem. 84, 1–15.
[9] Langan, T.A., Rall, S.C. and Cole, R.D. (1971) J. Biol. Chem. 246, 1942–1948.

[10] Langan, T.A. (1973) Adv. Cyclic Nucl. Res. 3, 99–153.
[11] Johnson, E.M. (1977) Adv. Cyclic Nucl. Res. 8, 267–309.
[12] Mende, L.M., Waterborg, J.H., Mueller, R.D. and Matthews, H.R. (1983) Biochemistry 22, 38–51.
[13] Charlesworth, M.C. and Parish, R.W. (1977) Eur. J. Biochem. 75, 241–250.
[14] Johns, E.W. (1964) Biochem. J. 92, 55–70
[15] Whitehouse, S. and Walsh, D.A. (1983) Methods Enzymol. 99, 80–93.
[16] Chambers, T.C. and Langan, T.A. (1990) J. Biol. Chem. 16940–16047.
[17] Heads, R.J. and Carpenter, B.G. (1990) Biochem. Biophys. Acta 1053, 56–62.
[18] Chambers, T.C., Song-Nichols, J., Campbell, D.S., Spitz, E., Leichting, B.H. and Rickenberg, H.V. (1987) Cell Diff. 20, 217–230.
[19] Laemli, U.K. (1970) Nature 227, 68–685.
[20] Panyim, S. and Chalkley, R. (1971) J. Biol. Chem. 246, 7557–7560.
[21] Langan, T.A. (1978) Methods Cell Biol. 19, 143–152.
[22] Woofendin, C., Chambers, T.C., Schaller, K.L., Leichtling, B.H. and Rickenberg, H.V. (1986) Dev. Biol. 115, 1–8.
[23] Chambers, T.C., Langan, T.A. Matthews, H.R. and Bradbury, E.M. (1983) Biochemistry, 22, 30–37.
[24] Langan, T.A. (1969) Proc. Natl. Acad. Sci. USA 64, 1276–1283.
[25] Harrison, J.J., Schwoch, G., Schweppe, J.S. and Jungman, R.A. (1982) J. Biol. Chem. 257, 13602–13609.
[26] Waterman, M. Murdoch, G.H., Evans, R.M. and Rosenfeld, M.G. (1985) Science, 229, 267–269.
[27] Ajiro, K., Shibata, K. and Nishikawa, Y. (1990) J. Biol. Chem. 265, 6494–6500.
[28] Suzuki, M. Sohma, H., Yazawa, M., Yagi, K. and Ebashi, S. (1990) J. Biochem. 108, 356–364.
[29] Watson, G. and Langan, T.A. (1973) Fed. Proc. 32, 588 (abstract).
[30] Felsenfeld, G. (1992) Nature 355, 219–224.
[31] Nacheva, G.A., Guschin, D.Y., Preoobrazhenskya, O.V., Karpov, V.L., Ebralidse, K.K. and Mirzabekov, A.D. (1989) Cell 58, 27–36.