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Kinetoplast DNA-aggregating proteins from the parasitic protozoan *Crithidia fasciculata*

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Proteins from *C. fasciculata*, which preferentially aggregate the parasite's AT-rich mitochondrial (kinetoplast) DNA in vitro, are reported. The aggregation is non-topological, non-covalent, independent of Mg^{2+} and ATP, but much improved by spermidine. We discuss how these proteins might modulate the shape and size of the parasite's mitochondrial genome.

DNA-aggregating protein; Kinetoplast DNA; *(Crithidia fasciculata)*

1. INTRODUCTION

The DNA (kDNA) in the single mitochondrion (kinetoplast) of kinetoplastid protozoans is made up of two types of catenated rings, the minicircles and maxicircles, and is organized into a tightly packed network as seen by electron microscopy and other studies [1–6]. The minicircle has a segment of bent helix which may contribute to this packaging [7] but DNA-binding proteins must also be relevant to the network's architecture and function [8]. For example, by modulating the shape and size of the network such proteins could allow polymerases and topoisomerases access to selected domains of the network at certain stages in the parasite's life cycle, such as during network replication [1]. Here I report a class of proteins from the parasitic protozoan *Crithidia fasciculata* which aggregates kDNA preferentially over nuclear and other DNAs, and discuss how these proteins might influence kinetoplast structure in vivo.

2. EXPERIMENTAL

2.1. *Parasites*

C. fasciculata stocks were kindly given by Dr Keith Vickerman, Glasgow, and were grown in Difco brain heart infusion medium supplemented with haemin (20 μg/ml) and gentamycin (75 μg/ml). Parasites, harvested in early stationary phase by centrifugation at 1800 × *g* for 20 min at 4°C, were washed once with PS buffer containing 10 g/l glucose and stored at −75°C until use.

2.2. *DNA*

Clones carrying maxicircle fragments and the minicircle are explained in fig.1a. pMaxD5D1 was kindly provided by Drs Rob Benne and Paul Sloof, Amsterdam. pMaxD3D4 and pMin56 were constructed in this laboratory in collaboration with Miss Ingrid Elfving. kDNA networks were prepared according to Simpson and Simpson [10] and nuclear DNA was prepared from supernatants in the same procedure. The DNA was deproteinized by several rounds of organic extraction, dialyzed against TE and stored at −15°C until use. DNA digested with restriction enzyme was dialyzed against TE before use. Synthetic polynucleotides (Pharmacia; made by random annealing of approximately 200 base long single strands) were dialyzed against TE + 50 mM NaCl before use.

2.3. *Protein extraction*

All operations were at 0–4°C. About 60 g wet wt of the parasites were resuspended to give 170 ml in buffer A (25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA and 12% (v/v) glycerol). This suspension was supplemented with 5 mM EDTA, 20 mM spermidine and 5% ammonium sulphate and disrupted in an Omni-Mixer (model 1706) in the presence of glass beads (Sigma G-9268). The 140 ml suspension recovered was slowly diluted to 250 ml with buffer A plus the supplements and centrifuged in a Sorvall SS34 rotor for 2 h at 15000 rpm. The supernatant (220 ml) was made 60% with ammonium sulphate and centrifuged in the Sorvall SS34 rotor for 20 min at 12000 rpm. 160 ml from the resulting supernatant were saturated with ammonium sulphate and the precipitate was collected by centrifugation in the SS34 rotor for 30 min at 15000 rpm. The pelletted material, dissolved in 13 ml buffer A (35 mg/ml protein), was applied to a 2.5 × 80 cm column of Sephacryl S-300 Superfine beads (Pharmacia) and eluted with buffer A at a flow rate of 75 ml/h. 5 ml fractions were collected and stored at −75°C until use. A representative chromatogram is shown in fig.1b.

2.4. *DNA aggregation assays*

Assay mixtures contained protein and DNA, in amounts and conditions given in the figure legends, in a total of 30 μl of buffer A + 5 mM spermidine. Protein was from pooled fractions 75 and 76 from the experiment shown in fig.1b. Assay mixtures were incubated in ice for 10 min, shifted to 30°C for 10 min and returned to ice. To each tube 6 μl of 6× loading buffer (0.25% Bromophenol blue/30% glycerol in water) were added and the contents electrophoresed at a constant voltage of 1 V/cm at room temperature in a 0.7% slab of agarose in TBE containing 0.2 μg/ml ethidium bromide.

2.5. *'South-Western' blots*

These were performed according to Staudt et al. [11].

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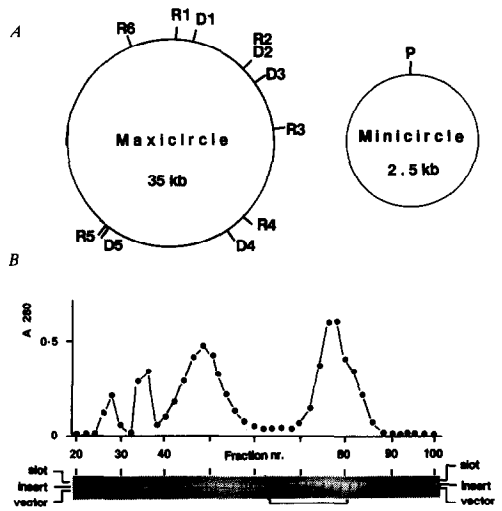

Fig.1. (A) Relevant restriction sites (approximately placed) on *C. fasciculata* maxicircle and minicircle. R, *Eco*RI; D, *Hin*dIII; P, *Pst*I. Maps are based on [9]. Clones used in this work: pMaxD3D4 is the D3D4 fragment (8 kb) cloned into the *Hin*dIII site in pBR322; pMaxD5D1 is the D5D1 fragment (12 kb) cloned into the *Hin*dIII site in pBR328; pMin56 is an entire *Pst*I cleaved minicircle cloned into the *Pst*I site in pBR322. (B) Resolution of kDNA aggregating activity by gel filtration. Graph shows optical density of fractions eluting from Sephacryl column. Picture below graph shows an agarose gel in which *Hin*dIII cleaved pMaxD5D1 DNA (see A) was electrophoresed after incubation with column fractions. About 1 μg of DNA and 15 μl of the appropriate column fraction were used in the assay. Conditions are given in section 2. Fractions 64–80, which span the aggregating activity, are underlined.

2.6. *Edman degradation*

This was performed in a 477A pulsed liquid phase sequencer (Applied Biosystems, California) with an on-line PTH 120A analyzer using regular cycle programmes and chemicals supplied by the manufacturer.

3. RESULTS

An extract from *C. fasciculata* (section 2.3; fig.1b), when used in low concentrations, retarded the electrophoretic mobility of both minicircle and maxicircle DNA much better than *C. fasciculata* nuclear DNA or bacterial plasmid DNA, although at higher concentrations it retarded all these DNAs equally well (fig.2a). The retardation was independent of Mg^{2+} and ATP (data not shown) but was much improved by 4 mM spermidine (and up to 20 mM tested) (fig.2b). Proteinase K (but not RNase) was able to restore the normal band intensity and migration pattern to the DNA, indicating that the retardation activity is a protein that does not join DNA molecules covalently. When supercoiled plasmids were used as substrate in the assay they were not catenated as judged by the absence of DNA in the gel slots following proteinase K treatment and electrophoresis, indicating that the protein is not a topoisomerase (fig.2c). The retarded DNA did not migrate as discrete bands but as a smear and at high concentrations of the protein it remained in the well despite prolonged electrophoresis, indicating that it is multimerized to form large aggregates. An end-to-end aggregation is unlikely as the DNA fragments did not form a 'ladder' at intermediate levels of the protein. More probably, the DNA molecules are aggregated along their length; the retardation of supercoiled DNA (fig.2c) supports this.

We wondered if the recognition motif for the protein was some aspect of the kDNA's high AT content (maxicircle 80% [12,13]; minicircle 57% [14]). Maxicircle DNA, with its higher AT content, indeed required much less extract for aggregation than did minicircle DNA (data not shown). In in vitro assays the synthetic DNA copolymer A-T·A-T and the homopolymer A·T competed successfully with kDNA for the aggregating activity, but the homopolymer G·C competed only with vector DNA. The alternating copolymers G-C·G-C, A-G·C-T, and A-C·G-T offered no detectable competition at the concentrations used (fig.3).

Aggregates formed by incubating the extract with different DNA species were sedimented by centrifugation to investigate proteins brought down in the pellet. Four proteins, p, q, r and s, with apparent M_r 22, 17, 15 and 14 kDa, respectively, sedimented in significantly higher amounts from incubations containing kDNA than from the control incubation containing only bacterial plasmid DNA (fig.4a). These proteins were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and probed separately with radioactive synthetic DNA polymers A-T·A-T or G-C·G-C, in the presence of a large excess of non-radioactive calf thymus DNA. The A-T·A-T probe bound to protein band q, while the G-C·G-C probe was competed out successfully by the calf thymus DNA (fig.4b).

Taken together, these observations suggest that proteins p, q, r and s form a multimer, or a family of multimers, which binds to AT-rich DNA via protein q. We cannot yet say if the protein recognizes a specific sequence of A's and T's or certain conformations of the DNA helix caused by an abundance of these bases [15–17]. We have not been able to obtain a clear foot print in DNA binding studies using a well-retarded 0.3 kb *Hin*dIII-*Rsa*I fragment from the D3R3 region of the maxicircle. This may be due to one or more of the following reasons: (i) The DNA-protein interaction is relatively weak; (ii) the protein binds to many sites along the DNA fragment; (iii) in aggregating the DNA, each protein multimer binds simultaneously to more than one DNA duplex at AT-rich, but not necessarily identical, sites.

The N-termini of proteins p, q and s were sequenced by Edman degradation. (The N-terminal end of protein r was found to be blocked.) When the SWISS-PROT protein sequence data base (Release 9; December 1988) was searched for homologies to subsequences of 7, 10 and 7 amino acids from proteins p, q and s, respectively, no homologies were found when mismatches were

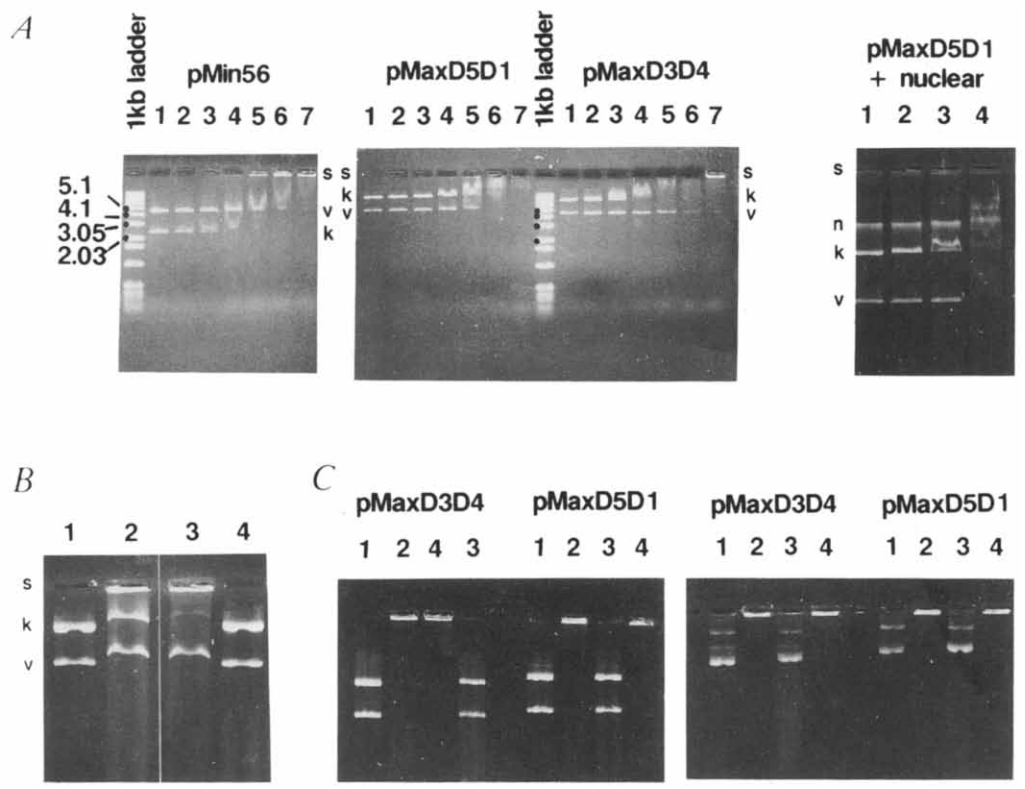

Fig.2. Agarose gel electrophoresis of DNA aggregates. For details see section 2.4. Except in C, right hand panel, linearized plasmid DNA (fig.1A) was used in all assays: pMin56 was cut with *Pst*I; pMaxD5D1 and pMaxD3D4 with *Hin*dIII. Lettering on side margins: s, gel slot; n, nuclear DNA; k, kDNA; and v, vector DNA. (A) Left centre: DNA (1.25 μg) was incubated with 0 μg (track 1), 0.15 μg (track 2), 0.3 μg (track 3), 0.6 μg (track 4), 1.2 μg (track 5), 1.8 μg (track 6) and 2.4 μg (track 7) of the protein. Right: Mixed incubation of 1.5 μg each of pMaxD5D1 DNA and *C. fasciculata* nuclear DNA with 0 μg (track 1), 0.3 μg (track 2), 0.6 μg (track 3) and 1.2 μg (track 4) of protein. (B) Effect of spermidine on retardation. Each incubation had 3 μg pMaxD5D1. Protein was dialyzed against buffer A before use at 1.8 μg per incubation. Track 1, DNA only; track 2, DNA + protein; track 3, DNA + protein + 4 mM spermidine; track 4, DNA + 4 mM spermidine. (C) Effect of proteinase K and ribonuclease A on DNA aggregates. After incubating the DNA/protein mixture, three equal aliquots were taken and incubated for a further 15 min at 30°C with either buffer alone, proteinase K (Merck; 100 μg/ml) or RNase (Sigma; 30 μg/ml) prior to electrophoresis in agarose. Each track represents an incubation of 2.5 μg DNA and, except for track 1, about 2.4 μg of protein. Track 1, DNA only; track 2, DNA + protein; track 3, DNA + protein + proteinase K; track 4, DNA + protein + RNase. Left: *Hin*dIII linearized plasmids. Right: Supercoiled plasmids; here the assay mixtures contained the following supplements to promote catenation: 20 mM KCl, 10 mM $MgCl_2$, 2.5 mM ATP and 20 μg/ml BSA. DNA and protein were incubated for 40 min before proteinase K or RNase treatment.

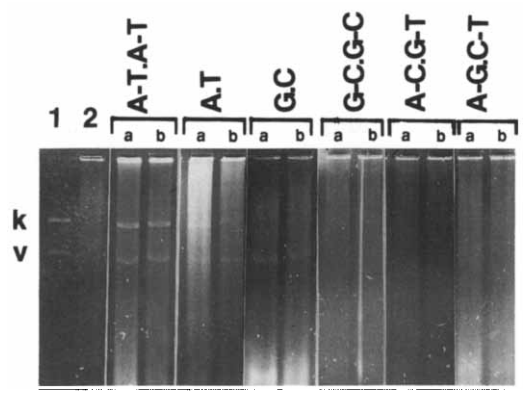

Fig.3. Aggregation of kDNA in the presence of synthetic polynucleotides. Assays were done as given in section 2. Each track represents an incubation of 1.5 μg *Hin*dIII digested pMaxD3D4 DNA and, except for the no-protein control run in track 1, 2.4 μg protein. Track 2, DNA + protein only. The polynucleotides, identified above the tracks, were included in the assay mixtures. Tracks marked 'a' and 'b' received incubation mixtures that had 24 μg and 8 μg of the polynucleotide, respectively. k, kDNA; v, vector DNA.

disallowed. Some published sequences showing a limited homology to these proteins are shown in fig.5.

4. DISCUSSION

The clear preference of this protein complex for AT-rich DNA suggests that it may be involved in the maintenance and modulation of kDNA network structure. The network contains some 25% of the cell's DNA in the form of about ten thousand catenated rings, the AT-rich maxicircles and minicircles. It has a highly organized structure, but is thought to vary its shape and size at certain stages in the parasite's life cycle, such as during kDNA replication [1], both to enable polymerases and topoisomerases to enter its matrix and to accommodate the increment of newly synthesized DNA. One way in which the proteins reported here might contribute to such a process is by anchoring the network to the mitochondrial mem-

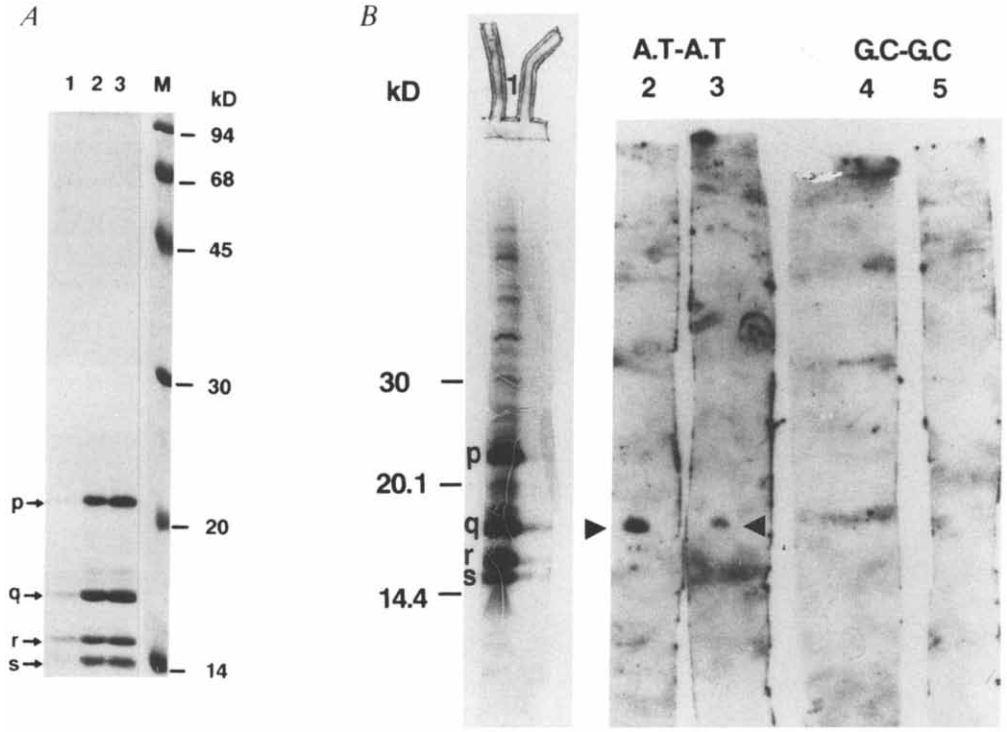

Fig.4. Proteins involved in kDNA aggregation. a, 3 × 1 ml aliquots (i.e. about 160 μg protein/aliquot) were taken from pooled fractions 75 and 76 (fig.1b), centrifuged briefly to remove denatured material and incubated with 90 μg of either supercoiled pBR328, supercoiled pMaxD5D1 or deproteinized whole kDNA networks in buffer A + 5 mM spermidine, under conditions given in section 2.4. After centrifuging the reaction tubes at 13000 rpm for 30 min at 4°C the supernatants were carefully removed. The proteins in the pellets were dissolved in Laemmli sample buffer, electrophoresed in a slab of 15% acrylamide and visualized by Coomassie blue staining. Track 1, pBR328; track 2, pMaxD5D1; track 3, kDNA networks. b, South-Western blot of proteins. Equal amounts of kDNA/protein aggregates obtained in experiments similar to that given in a were separated in several tracks of the same slab gel by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was treated [11] to renature the blotted proteins, and cut into individual tracks. The strips of membrane were hybridized with ^{32}P labelled synthetic DNA polymers (identified above the tracks) in the presence of excess calf thymus DNA as described previously [11] and exposed to film. Track 1, Coomassie blue stained/dried gel strip; tracks 2–5, radioactivity in strips of nitrocellulose membrane. Probe (about 5 μg DNA per strip of membrane) contained about a million cpm/ml in each case. Tracks 2 and 4, probe + 500 μg unlabelled calf thymus DNA; tracks 3 and 5, probe + 2000 μg unlabelled calf thymus DNA. The radioactivity in bands corresponding to protein q in tracks 2 and 3 is arrowed.

	amino terminal end
protein p	? T P ? S A P R K A A
	S A v R K A A
protein q	? T P ? S A K K A S R K S G
	S A g K p S R K S e
protein s	? K G H ? S A D A K G S
	S m D A K G S

Fig.5. N-terminal sequences of proteins p, q and s obtained by Edman degradation. kDNA-protein complexes were separated by SDS-PAGE as shown in fig.4a, electroblotted onto an Imobilon-P PVDF transfer membrane (Millipore) and stained with Coomassie blue using procedures recommended by the manufacturers. Bands corresponding to proteins p–s were excised from the membrane and used for amino acid sequence determination (see section 2). The one-letter amino acid code is used. Question marks are ambiguous residues. Row below each trypanosome protein sequence is a partially matching sequence obtained by searching the SWISS-PROT data base; mismatches are in lower case letters. The partially matching sequences were found in the following proteins. For protein p: the probable DNA polymerase from Hepatitis B virus [18]. For protein q: the giant secretory protein I-A from midge [19]. For protein s: haptoglobin from rat liver [20].

brane, rather like the nuclear scaffold-DNA interactions proposed for *Drosophila* tissue culture cells [21] and the envelope-chromosome attachments proposed for bacteria [22]. Note here the supportive evidence that maxicircles, the most AT-rich component of the kDNA, are found in the periphery of the network for much of the trypanosome life cycle [23]; and that the histone-like HU protein of bacteria appears to be gathered towards the periphery in isolated nucleoids [24]. Another way the proteins might influence network movement is by connecting the catenated rings at suitable sites of helical geometry, but allowing them to slip now and then to occupy a hierarchy of secondary sites, causing the network as a whole to undergo a process of 'breathing'. Polyamines have been reported in various organisms including *Crithidia* and their levels have been shown to fluctuate during the cell cycle [25–27]. We may therefore reasonably expect polyamines to play a part in modulating these kDNA-protein interactions in vivo, as we have found them to do in vitro (fig.2b).

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REFERENCES

[1] Ryan, K.A., Shapiro, T.A., Rauch, C.A. and England, P.T. (1988) Annu. Rev. Microbiol. 42, 339–358.
[2] Stuart, K. (1983) Mol. Biochem. Parasitol. 9, 93–104.
[3] Simpson, L. (1987) Annu. Rev. Microbiol. 41, 363–382.
[4] Delain, E. and Riou, G. (1969) CR Acad. Sci. Paris Ser. D 268, 1225–1227.
[5] Simpson, L. and Da Silva, A. (1971) J. Mol. Biol. 56, 443–453.
[6] Silver, L.E., Torri, A.F. and Hajduk, S.L. (1986) Cell 47, 537–543.
[7] Marini, J.C., Levene, S.D., Crothers, D.M. and Englund, P.T. (1982) Proc. Natl. Acad. Sci. USA 79, 7664–7668.
[8] Tittawella, I. (1989) FEMS Microbiol. Lett. 60, 347–352.
[9] Hoeijmakers, J., Schoutsen, B. and Borst, P. (1982) Plasmid 7, 199–209.
[10] Simpson, A.M. and Simpson, L. (1974) J. Protozool. 21, 774–781.
[11] Staudt, L.M., Clerc, R.G., Singh, H., Le Bowitz, J.H., Sharp, P.A. and Baltimore, D. (1988) Science 241, 577–580.
[12] Sloof, P., Van den Burg, J., Voogd, A. and Benne, R. (1987) Nucleic Acids Res. 15, 51–65.
[13] Sloof, P., Van den Burg, J., Voogd, A., Benne, R., Agostinelli, M., Borst, P., Gutell, R. and Noller, H. (1985) Nucleic Acids Res. 13, 4171–4190.
[14] Birkenmeyer, L., Sugisaki, H. and Ray, D.S. (1985) Nucleic Acids Res. 13, 7107–7118.
[15] Nelson, H.C.M., Finch, J.T., Luisi, B.F. and Klug, A. (1987) Nature 330, 221–226.
[16] Solomon, M.J., Strauss, F. and Varshavsky, A. (1986) Proc. Natl. Acad. Sci. USA 83, 1276–1280.
[17] Traverse, A.A. (1989) Annu. Rev. Biochem. 58, 427–452.
[19] Valenzuela, P., Gray, P., Quiroga, M., Zaldivar, J., Goodman, H.M. and Rutter, W.J. (1979) Nature 280, 815–819.
[19] Wieslander, L., Sumegi, J. and Daneholt, B. (1982) Proc. Natl. Acad. Sci. USA 79, 6956–6960.
[20] Goldstein, L.A. and Heath, E.C. (1984) J. Biol. Chem. 259, 9212–9217.
[21] Mirkovitch, J., Mirault, M. and Laemmli, U.K. (1984) Cell 39, 223–232.
[22] Jacob, F., Brenner, S. and Cuzin, F. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 329–348.
[23] Englund, P.T., Hajduk, S.L. and Marini, J. (1982) Annu. Rev. Biochem. 51, 695–726.
[24] Bjornsti, M.A., Hobot, J., Kelus, A., Villiger, W. and Kellenberger, E. (1985) in: Bacterial Chromatin (Gualerzi, C., ed.) Springer, New York.
[25] Bacchi, C.J. and McCann, P.P. (1987) in: Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies (McCann, P.P., Pegg, A.E. and Sjoerdsma, A. eds) pp.317–354, Academic Press, Orlando, FL.
[26] Tabor, C.W. and Tabor, H. (1984) Annu. Rev. Biochem. 53, 749–790.
[27] Pegg, A.E. (1986) Biochem. J. 234, 249–262.