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Different superstructural features of the complexes between spermine and the light responsive elements of the two pea genes rbcS-3A and rbcS-3.6. Gel electrophoresis and circular dichroism studies

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Abstract

Two light responsive elements (LREs), DNA sequences, 62 base pairs long, relevant to the light control during transcription of the pea genes rbcS-3A and rbcS-3.6, were synthesized and ligated to obtain multimers with defined superstructural features. Their gel electrophoretic mobilities were studied in the presence of the tetracation, spermine, since it was previously suggested, on the basis of theoretical analysis, that spermine can increase DNA bending and thus could be useful in revealing DNA superstructural features. In fact, the difference between the curvatures of the two LREs, derived from gel electrophoresis retardation ratios, increases in the presence of spermine. Circular dichroism spectra of the complexes between spermine and the two LREs, at different neutralization ratios, show that the polyamine is able to induce the formation of asymmetric arrangements of complexes of molecules. The chirality of these complexes appears dramatically different for the two LREs, suggesting that their different superstructural features give rise to different interactions with the polyamine.

Keywords: DNA superstructures; DNA–spermine complexes circular dichroism; DNA–spermine complexes electrophoretic mobilities; rbcS genes; Light responsive elements

1. Introduction

It is now generally accepted that DNA superstructural features are relevant in specific interactions with proteins, involved in the regulation of important biological processes, such as transcription, recombination and chromatin organization [1–6]. In most cases DNA curvature appears to derive from nucleotide sequences and to be increased by specific binding with proteins [7,8]. While the role of

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proteins either on inducing or stabilizing DNA superstructures is well recognized and in some cases also structural periodicity and preferred configuration of DNA in DNA–protein complexes are known [9], other factors relevant in establishing DNA superstructures and in determining their stability and dynamics, such as binding to specific ions, have but scarcely been explored.

Diekmann and Wang [10,11] found that bent DNAs were further retarded when "electrophoresed" in the presence of 10 m*M* Mg^{2+}. Furthermore, Laundon and Griffith [12] have shown, by electron microscopy visualization, that a DNA segment of about 200 base pairs from *Crithidia fasciculata* kinetoplast minicircles bends into a small circle. The fraction of molecules with the most extreme bend increased from about 2% to 50–60%, following incubation of the DNA with increasing concentrations of divalent cations such as Zn^{2+}, Co^{2+}, Ba^{2+}, and Mn^{2+}. This behaviour could depend on the formation of metal complexes with DNA phosphates stabilized in the case of Mn^{2+} and Co^{2+} by the reaction with N7 of guanine. However, aggregation of DNA and precipitation of metal on the electrodes have precluded from studying the increase of DNA curvature by gel electrophoresis.

In this research we have chosen to study the influence on DNA curvature of the tetracation spermine, which is biologically of interest, since it is an important regulator of cell growth and differentiation [13]. Furthermore, theoretical predictions as well as studies of polyamine–nucleic acid systems in crystal and in solution state, have shown that spermine is able to induce many different conformational changes in DNA [14].

The curved DNA sequences studied are two 62-bp long DNA tracts positioned between −170 and −109 from the start-site of transcription of the two pea genes rbcS-3A and rbcS-3.6, encoding for the small subunit of ribulose-1,5-bisphosphate carboxylase. These two DNA sequences, named light responsive elements, LREs, are necessary for the light control on the transcription of rbcS-3A and rbcS-3.6. We have previously [15,16] carried out the study of the superstructural features of the two LREs as well as of the five different 21-bp long DNA tracts in which they can be dissected (one tract is shared by the two LREs). This group of different sequences with different curvatures offers the opportunity to study the influence of spermine on DNA superstructural features in a biologically relevant system.

2. Materials and methods

Oligodeoxyribonucleotides were synthesized on a Biosearch DNA synthesizer and purified on a 20% polyacrylamide gel in the presence of 7 *M* urea, followed by high salt (0.1 *M* ammonium bicarbonate) elution from a Sephadex G-50 column. Oligomer sequences were verified by using the chemical sequencing technique of Maxam and Gilbert with minor modifications for short oligomers [17]. Oligonucleotides were phosphorylated, and complementary sequences were annealed as previously described [18]. Polydeoxyribonucleotides were obtained by adding to 10 μl of the hybridized mixture 1 unit of T4 DNA ligase in the case of 21 mers and about 0.1 unit in the case of 62 mers. The reaction solution contained 70 m*M* Tris-HCl (pH 7.6), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol (DTT) and 2.3 m*M* cold ATP. The ligation reaction was allowed to proceed on ice overnight in the case of 21 mers, and at 4°C for 15 minutes in the case of 62 mers.

Ligated products were run on non denaturing 10% polyacrylamide gels (mono-to-bis acrylamide ratio = 29:1; 90 m*M* Tris-borate, pH 8.3) until the bromophenol blue dye had migrated 30 cm, in the absence of spermine, or 40 cm with spermine present in the gel and in the running buffer. The applied field was 4 V/cm. All electrophoresis experiments were carried out at room temperature. With spermine present in the running buffer, the buffer was pumped between the electrodes compartments with a rate of 5 l/hour each way. For higher currents during electrophoresis, electrode processes strongly influence DNA migration in the gel as well as the gel matrix itself. These influences become negligible when the buffer is exchanged between the electrode compartments at high rate.

Salmon sperm DNA was purchased from Sigma and used after phenol/chloroform extraction and alcohol precipitation to eliminate protein contamination. Its base-pair length was estimated by gel electrophoresis to be about 7000 bp.

Nucleosomal DNA was prepared from chicken erythrocytes by a modification of the method of Lutter [19], as previously reported [20]. The DNA molecular weight was checked by agarose gel electrophoresis and was found to average 170 bp in size.

Spermine · 4HCl was purchased from Fluka and further purified by recrystallization from 90% ethanol. The salt was stored under dessication until use. Solutions of spermine 0.1 *M* were prepared in the same buffer to be used in gel electrophoresis or in circular dichroism measurements.

Circular dichroism (CD) spectra were obtained with a Jasco model J 500 A dichrograph. The instrument was standardized by times with a solution of 10 (+) camphorsulfonic acid (1 mg/ml in water). The CD spectra were obtained in a 1.0 cm pathlength quartz cell, where the oligonucleotides concentrations fall within 0.4–0.5 OD units/ml.

The complexes between the oligonucleotides and spermine were prepared by slow mixing. The ratios between the amino groups of spermine and the DNA phosphates were suitably chosen to make the complexes soluble in aqueous buffer with a turbidity at 310 nm never exceeding 10% of the value of absorbance at 260 nm (namely $A_{310} \leq 0.05$). Furthermore the spectra showed a good reproducibility, ruling out relevant effects on CD features due to the slight turbidity of spermine–DNA complexes solutions, at the highest neutralization ratio.

3. Results

3.1 Use of electrophoresis gel retardation to study spermine–DNA interactions

The gel electrophoretic mobilities of the two LREs of the pea genes rbcS-3A and rbcS-3.6 were studied in the presence of different spermine concentrations in the samples as well as in the electrophoresis buffer. In all cases, gel retardations increased up to a spermine concentration of 0.1 m*M* and afterward remained constant. For this reason only the retardation values corresponding to spermine concentration equal to 0.1 m*M* are reported. In Fig. 1(a) a typical polyacrylamide gel is shown, where the retardation of the multimers obtained by ligation of the two LREs with respect to the standard straight sequence is clearly evident. On this gel, the retardation of rbcS-3A LRE appears slightly higher than that of rbcS-3.6 LRE.

The migration patterns from this and similar gels (at least three independent experiments were carried out in each case) are reported as the ratio (*R*) between the apparent and the true number of base pairs versus the true number of base pairs. It must be taken into account that, although the variations of *R*-values for a particular sequence in different experiments are about ± 0.02, the differences between the *R*-values of two sequences ($\Delta R = R_{\text{seq. I}} - R_{\text{seq. II}}$) in the same experiment, show better reproducibility (± 0.01). In fact small differences in factors such as gel reticulation, temperature, and buffer, seem to influence equally the mobilities of different sequences. In Fig. 1(b) the *R*-values of the two LREs are reported, showing that rbcS-3A LRE curvature is higher than that of rbcS-3.6 LRE, as previously suggested on the basis of theoretical analysis [18]; in both cases, the polyamine increases the retardation, and seems also to slightly increase the difference between the two sequences, as shown in Fig. 1(c).

These findings are substantiated by the study of the spermine influence on the retardations of the three boxes in which each of the two LREs can be divided on the basis of biochemical and genetic analyses [21].

In a previous paper [16] we have studied the superstructural features of the three boxes by gel electrophoretic retardation analysis and found that both LREs present a superstructure organized as two

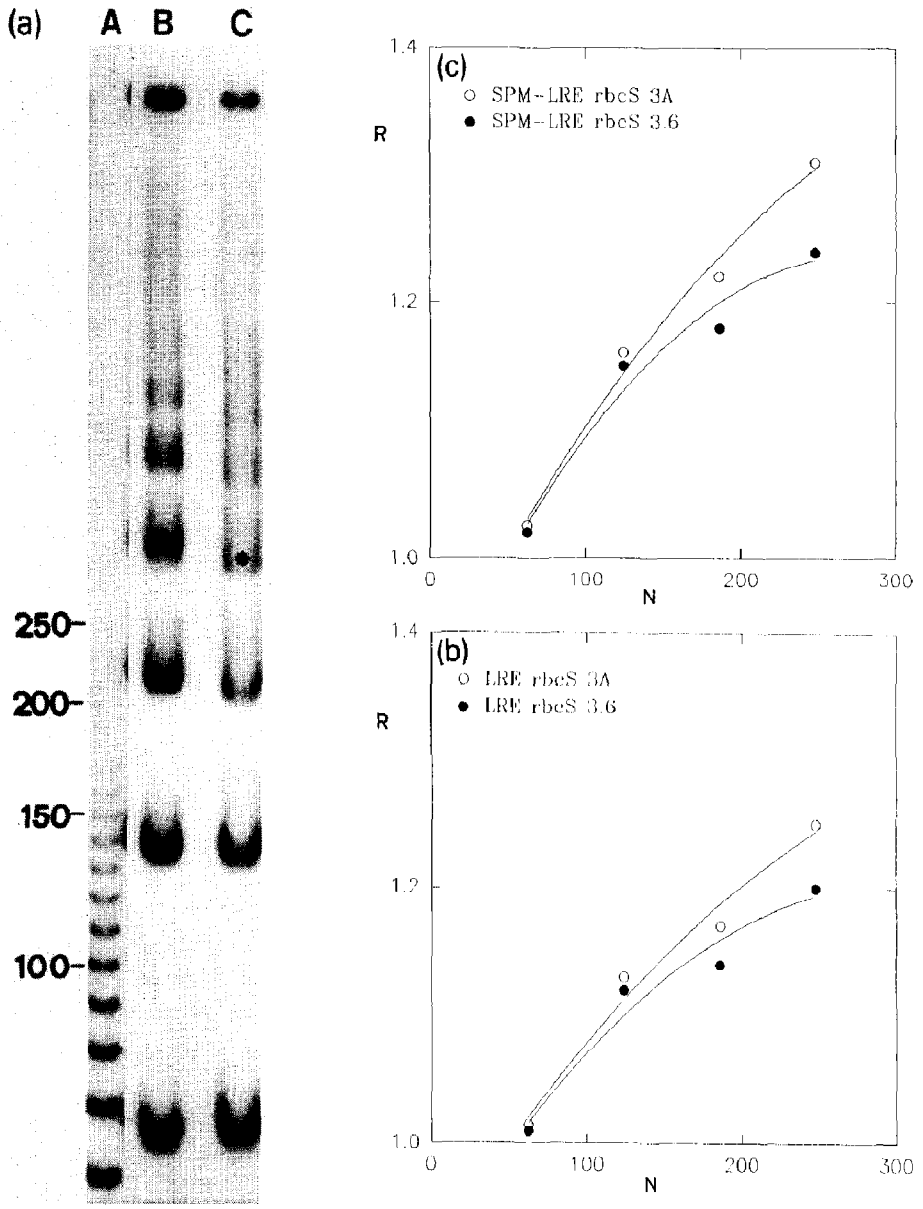

Fig. 1. (a) Autoradiogram of the multimer series (derived from the rbcS-3A and rbcS-3.6 LRE sequences reported in Table 1) electrophoresed on a non-denaturing 10% polyacrylamide gel at room temperature. Lane A, NBH; lane B, rbcS-3A LRE; lane C, rbcS-3.6 LRE. Asterisks indicate multimers of 248 bp length. (b) The ratio (R) between apparent and real molecular weight, determined from comparison with the electrophoresis markers, are plotted as function of N (the actual chain length). (c) R-values vs. N, in the presence of 0.1 mM spermine.

curved tracts, connected by a straight tract. While the straight tract, box II, is equal in the two cases, the other two tracts box I and box III present a higher curvature in the case of the LRE of the rbcS-3A gene, in agreement with theoretical prediction. In Fig. 2(a) (left side) the autoradiogram of the multimer series obtained by ligation of the boxes I are shown. The higher curvature in the case of the sequence, belonging to rbcS-3A gene, is clearly evident, while the sequence of box II is practically straight.

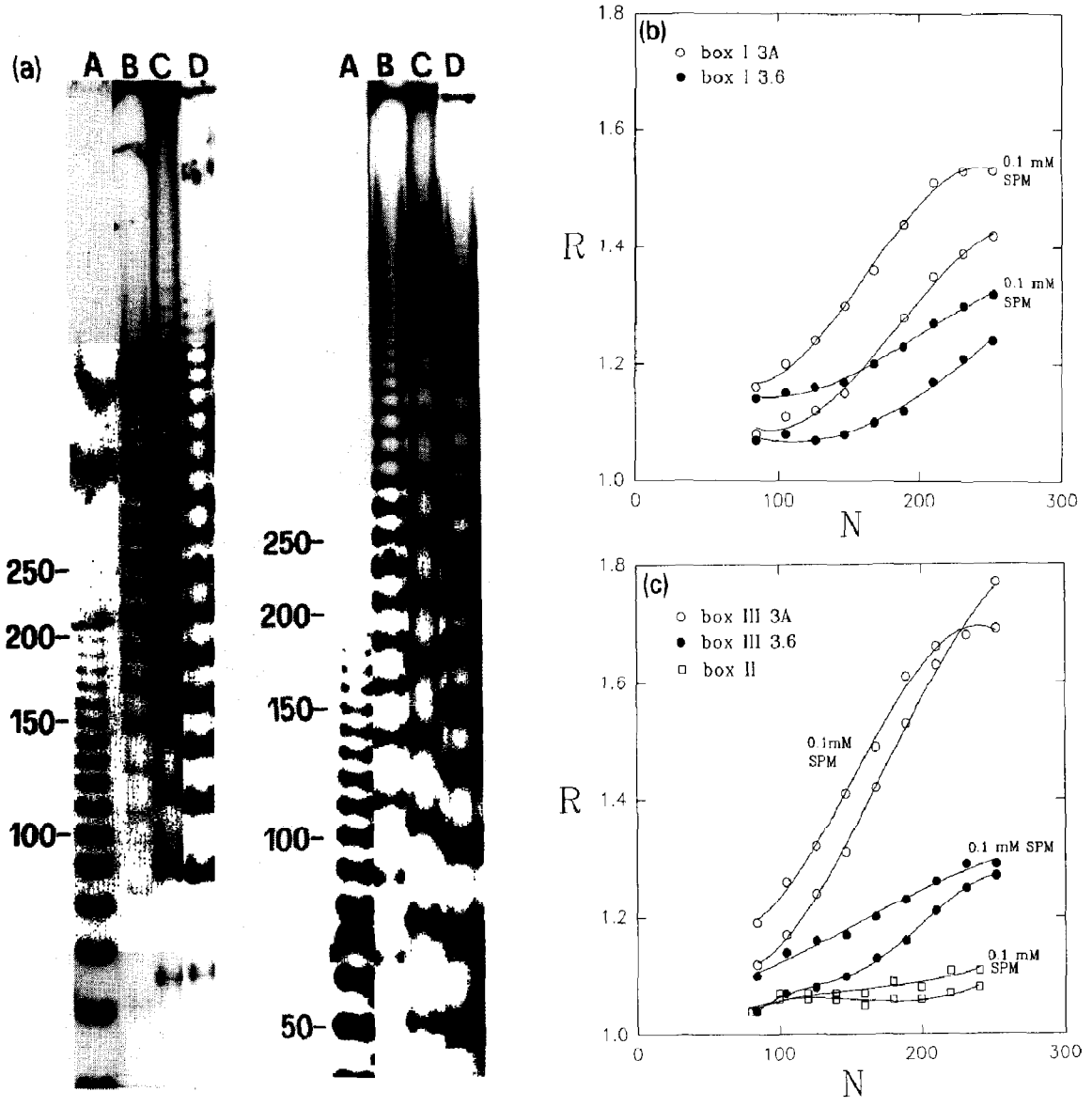

Fig. 2. (a) Autoradiogram of the multimer series, deriving from boxes I of the two LREs of the rbcS-3A and rbcS-3.6 genes and from box II (the sequences are reported in Table 1) electrophoresed on a non-denaturing 10% polyacrylamide gel at room temperature. Left side without spermine, right side in the presence of spermine 0.1 m*M*. Lane A, NBH; lane B, box II; lane C, box I 3A; lane D, box I 3.6. (•) indicate multimers of 200 bp length. (*) indicate multimers of 210 bp length. (b) *R*-values of boxes I vs. *N* in absence and in presence of 0.1 m*M* spermine. (c) *R*-values of boxes II and boxes III vs. *N* in absence and in presence of spermine 0.1 m*M*.

If spermine 0.1 m*M* is added, the difference between the two boxes I increases, (see Fig. 2(a) right hand side), while the behaviour of box II is only slightly affected by the polyamine. Also the retardation of boxes III increases in the presence of spermine (not shown).

A more quantitative evaluation of spermine influence on the retardation of the different boxes is obtained by plotting *R* versus *N* (the actual chain length). Figure 2(b) shows that the retardations of boxes I of both genes as well as their differences are strongly increased by the polyamine. Figure 2(c)

similarly shows that the retardations of boxes III are increased in the presence of spermine, although the polyamine influence seems less relevant. However, also in this case the differences between R-values in the presence of spermine are well above the expected errors. The retardation of box II is only slightly influenced by the polyamine.

3.2 Circular dichroism spectra of the complexes between spermine and the LREs of the rbcS-3A and rbcs-3.6 genes

Circular dichroism spectra (CD) of the complexes between the LREs of the two genes and spermine were carried out with the aim of evaluating the possible influence of the polyamine on LREs conformations. CD spectra of the two LREs in Tris 10 mM buffer at pH 7.5, show that the two DNA elements are in the standard B conformation; minor differences with respect to the CD spectra of eukaryotic mixed sequences DNAs in the same salt conditions can be attributed to the effect of different nucleotide sequences on DNA structural parameters [22].

The addition of spermine to the LRE solutions, either in the case of rbcS-3A LRE or rbcS-3.6 LRE, increases the positive band and decreases the negative one, although with different trends (Figs. 3(a) and 3(b)). This different behaviour can be more easily evaluated in Fig. 3(c), where the ellipticities at 280 nm are reported versus the neutralization ratios, namely the ratio between spermine aminogroups and DNA phosphates (r_{SPM}). This plot shows that a larger neutralization ratio is necessary in the case of rbcS-3.6 LRE with respect to rbcS-3A to obtain the same increase of the positive band: this result may reflect a difference of binding constant and/or a difference of polyamine effect on the complexes. The influence of spermine on the CD spectra was studied decreasing the ionic strength of the buffer, since lowering the competition of Tris ions, the different features of the polyamine binding to the two LREs could become more evident. In fact the complexes, at ionic strength corresponding to Tris 5 mM, show a dramatically different behaviour. Increasing the neutralization ratio (r_{SPM}), the CD spectra of the rbcS-3A LRE present an increase of the positive band and a decrease of the negative one, similarly than in Tris 10 mM (Fig. 4(a)). On the contrary the rbcS-3.6 spectra are characterized by a red-shift (about 10 nm) of the maximum at 280 nm, the decrease of the positive band and the increase of the negative one, as shown in Fig. 4(b). This behaviour, that can be described naively as opposite, is more evident if the ellipticities at 280 nm are reported versus r_{SPM} (Fig. 4(c)).

At further decreasing of ionic strength, the CD features become similar to those in Tris 10 mM. In fact, as it is shown in Figs. 5(a) and Sb, in both cases increasing r_{SPM} the positive band increases, while the negative band decreases.

3.3 Circular dichroism spectra of complexes between spermine and nucleosomal DNA

The influence of DNA molecular weight on the complexation reaction with spermine was evaluated by examining the features of the CD spectra of the complexes between the polyamine and two eukaryotic DNAs of very different molecular weight: namely nucleosomal DNA, about 170 bp long, and sperm salmon DNA, about 7000 bp long. The CD spectra of the complexes between spermine and nucleosomal DNA, at increasing r_{SPM}, are reported in Fig. 6(a). The behaviour is very similar to that of rbcS-3A LRE in the same salt conditions. In fact, the positive band at 280 nm increases with increasing r_{SPM}, while the negative band, at about 240 nm, strongly decreases.

The behaviour of the complexes between the polyamine and salmon sperm DNA is noticeably different. As shown in Fig. 6(b), at increasing r_{SPM}, only a slight decrease of the positive band is apparent, while the negative band appears almost unchanged.

The different behaviour of the two DNAs is more easily observable in Fig. 6(c), where the ellipticities at 280 nm are reported versus r_{SPM}.

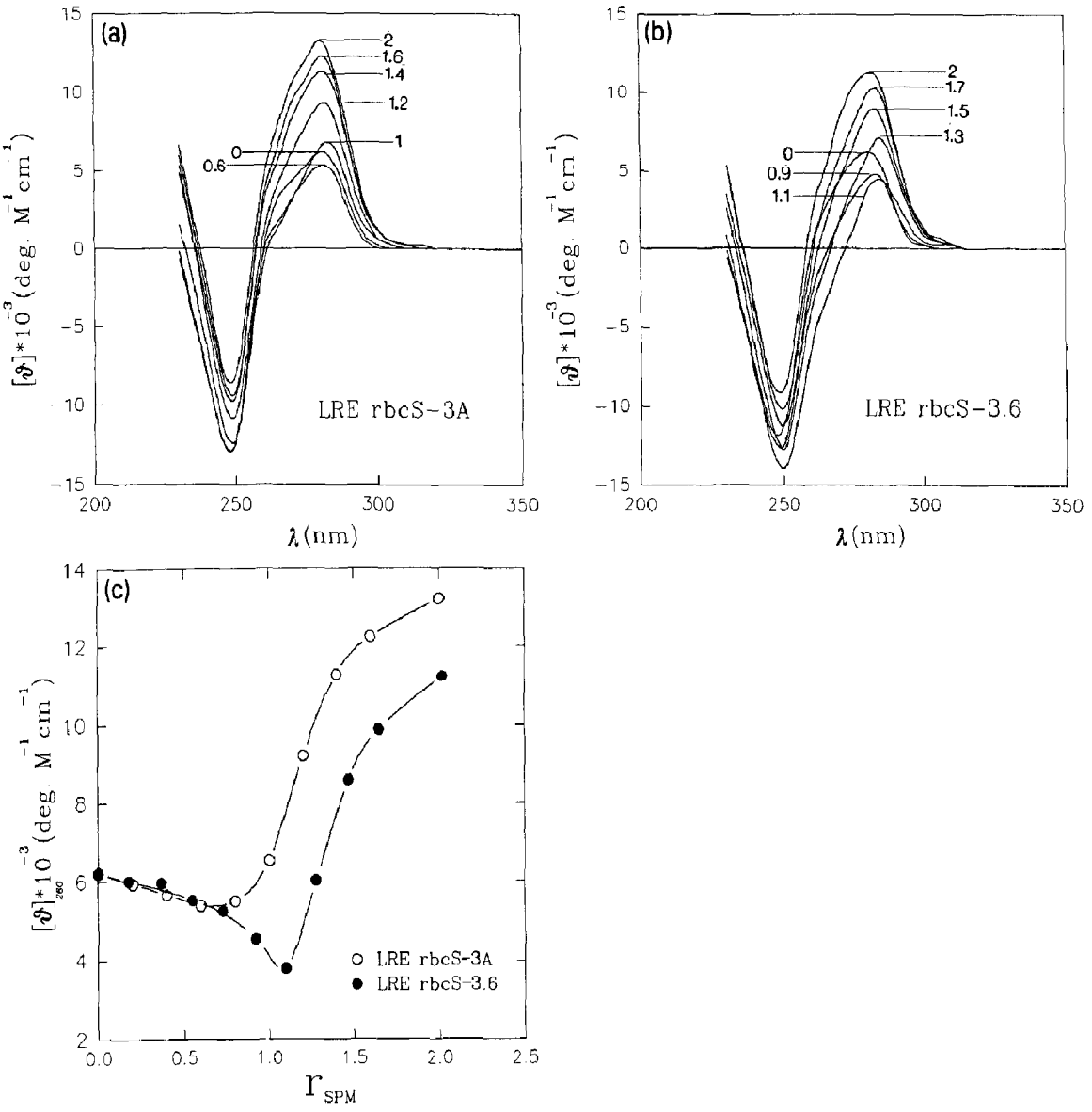

Fig. 3. Circular dichroism (CD) spectra of the complexes between spermine and (a) the LRE of the rbcS-3A gene, and (b) the LRE of the rbcS-3.6 in 10 m*M* Tris pH 7.5, at increasing ratios between polyamine amino groups and DNA phosphates (r_{SPM}). r_{SPM} values are reported in the figures. In (c) the molar ellipticities [ϑ] (at $\lambda = 280$ nm) vs. r_{SPD} are reported.

These results suggest that the formation of asymmetric arrangements of complexes between DNA and polyamine requires a low molecular weight.

4. Discussion

The main feature emerging from the electrophoretic experiments, is the increase of the retardation ratios, *R*, with increasing polyamine concentration up to 0.1 m*M*, for all the curved sequences examined;

only in the case of the sequence corresponding to box II, a straight DNA fragment shared by the two LREs, the electrophoretic mobility does not change significantly in the presence of spermine. It is worth noting that the spermine binding depends on DNA curvature in the case of two highly homologous sequences as the two LREs, hereafter reported in a way to put in evidence their homology

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LRE-3A   -170 ACACAAAA..TTTCAAATCTTGTGTGGTTAAT _141
              ||||| ||  |||||| ||||||||||||||||
LRE-3.6  -170 ACACACAACTTTTCAA.TCTTGTGTGGTTAAT _140
LRE-3A   -140 ATGGCTGCAAACTTTATCATTTTCACTATCTA _109
              ||||||||||| |||||||||| ||| |||||
LRE-3.6  -139 ATGGCTGCALAGTTTATCATTT.CACAATCTA _109
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This is in very good agreement with the theoretical model of spermine binding to DNA, previously proposed by Feuerstein et al. [14,23]. These authors used molecular dynamics to model interactions between spermine and DNA, and found that spermine bound to bent DNA is more stable than spermine bound to unbent DNA sequences. This model accounts also for the spermine influence on the retardations of the two boxes I; in fact the R-values increase in both cases and the increase is larger in the case of box I of the rbcS-3A LRE, which is more curved. The polyamine influence is less evident on boxes III electrophoretic mobilities, although boxes III present a curvature comparable to that of boxes I. This finding could depend on polyamine specific recognition of different sequences geometry.

According to Feuerstein et al. [14], spermine should interact better in the major groove of alternating purine/pyrimidine sequences, resulting in substantial DNA bending.

Hereafter the sequences of box I and box III of rbcS-3A are reported to put in evidence tracts of alternating purine/pyrimidine

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box I   3A PuPyPuPyPuPyPuPuPuPuPyPyPyPyPuPuPuPyPyPyPy
box III 3A PyPuPuPuPyPyPyPyPuPuPyPyPuPuPyPyPyPyPuPuPyPyPu
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It can be observed that a tract of PuPy six bp long (underlined), sufficient to assure a stereospecific strong binding of one spermine molecule, is present only in boxes I and thus could be relevant in the different spermine influence on the retardation of boxes I and boxes III. obviously this explanation implies a highly stereospecific binding of the polyamine to different sequences; on this topic it is interesting to remember the recent findings [24] obtained by X-ray diffraction on crystals of the ternary complex between spermine, DNA and 4′-epiadriamycin, showing that the polyamine is able to discriminate between AT and TA sequences.

Finally, the trends of the retardation ratios versus N of the different sequences examined and of their complexes with spermine was evaluated by plotting the ratio between the retardation of the complexes and the retardation of the different sequences, R_{SPM}/R, versus N. As shown in Fig. 7(a), in the case of the LREs, R_{SPM}/R-values increase up to N equal to 186 and then seems to remain constant. In the case of the boxes, the trend of R_{SPM}/R is similar, although the maxima correspond to different N-values. The results suggest that in all cases the complexes reach the plateau, due to the formation of one superhelical turn, at lower N-values with respect to the uncomplexed DNA. This could derive by the increase of helical twist due to the polyamine binding, in agreement with the influence of Mg^{2+} ions on retardation, as previously suggested [25].

Circular dichroism (CD) is a convenient method to assay DNA conformation in solution and to evaluate the influence of different chemical and physical factors. The CD spectra of the two LREs show that the two DNA elements are both in B-type conformation. Minor differences, with respect to the CD spectra of eukaryotic mixed sequence DNAs, such as the smaller rotational strength of the positive band with respect to that of the negative one, could derive from variations of structural parameters of DNA double helix such as major groove width, rise per base pair, base pair tilt, on account of different sequences as extensively discussed by Rhodes and coworkers [22].

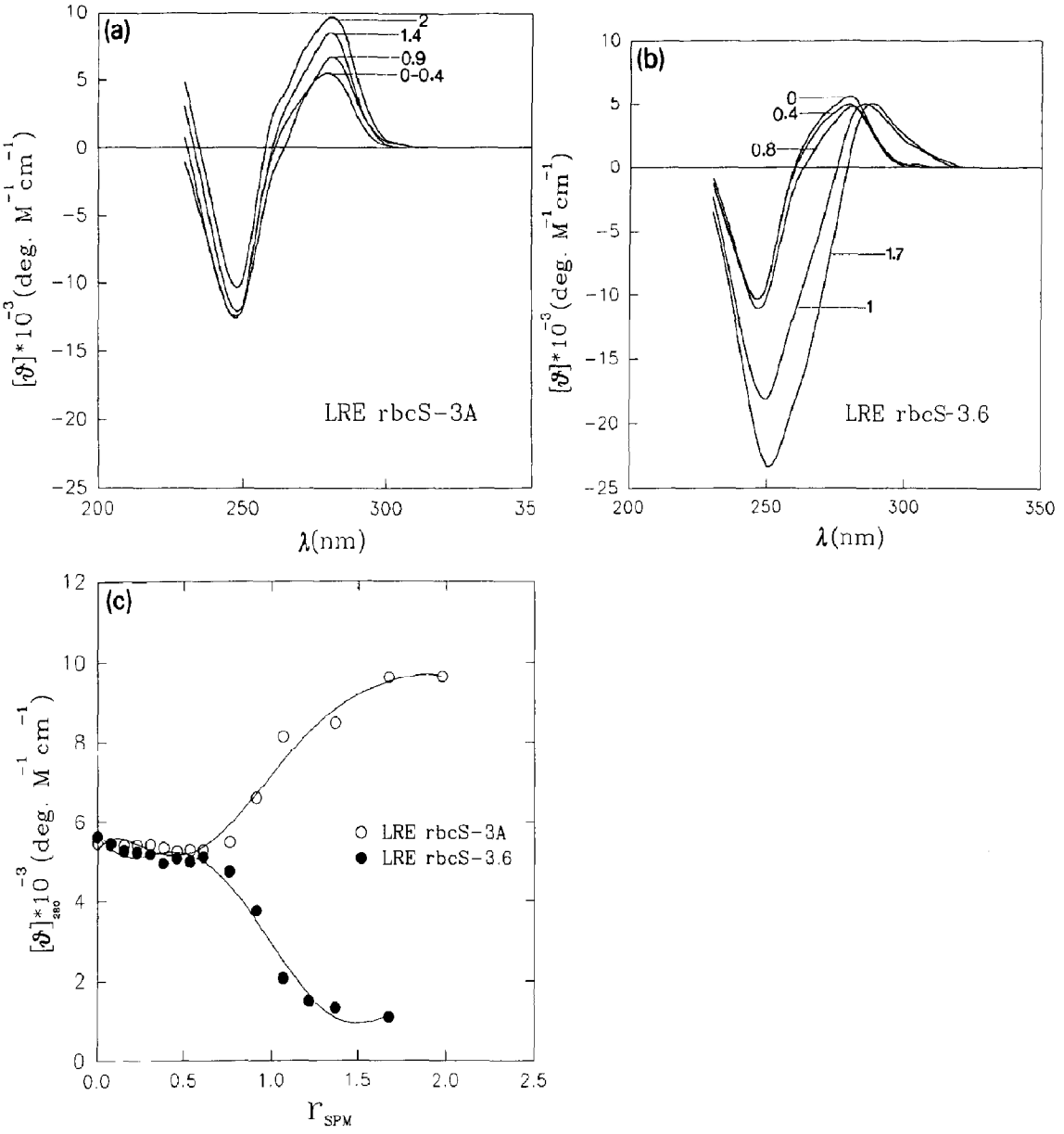

Fig. 4. CD spectra of the complexes between spermine and (a) the LRE of the rbcS-3A gene and (b) the LRE of the rbcS-3.6 in 5 m*M* Tris pH 7.5 at increasing values of r_{SPM}, as reported in the figures. In (c) the molar ellipticities $[\vartheta]$ (at $\lambda = 280$ nm) vs. r_{SPM} are reported.

The interactions between LREs and spermine induce large variations in the CD spectra; these variations increase raising the neutralization ratios between the spermine aminogroups and the DNA phosphates and depend on the ionic strength of the solutions. We have examined the complexes at three different ionic strengths, with the aim of putting in evidence the features of spermine binding, decreasing the competition of Tris ions. The results obtained can be summarized as follows: (i) The CD spectra of the LRE of rbcS-3A gene, which is the more curved, are characterized, at all the ionic strengths examined, by the increase of the positive band and the decrease of the negative one at increasing r_{SPM}.

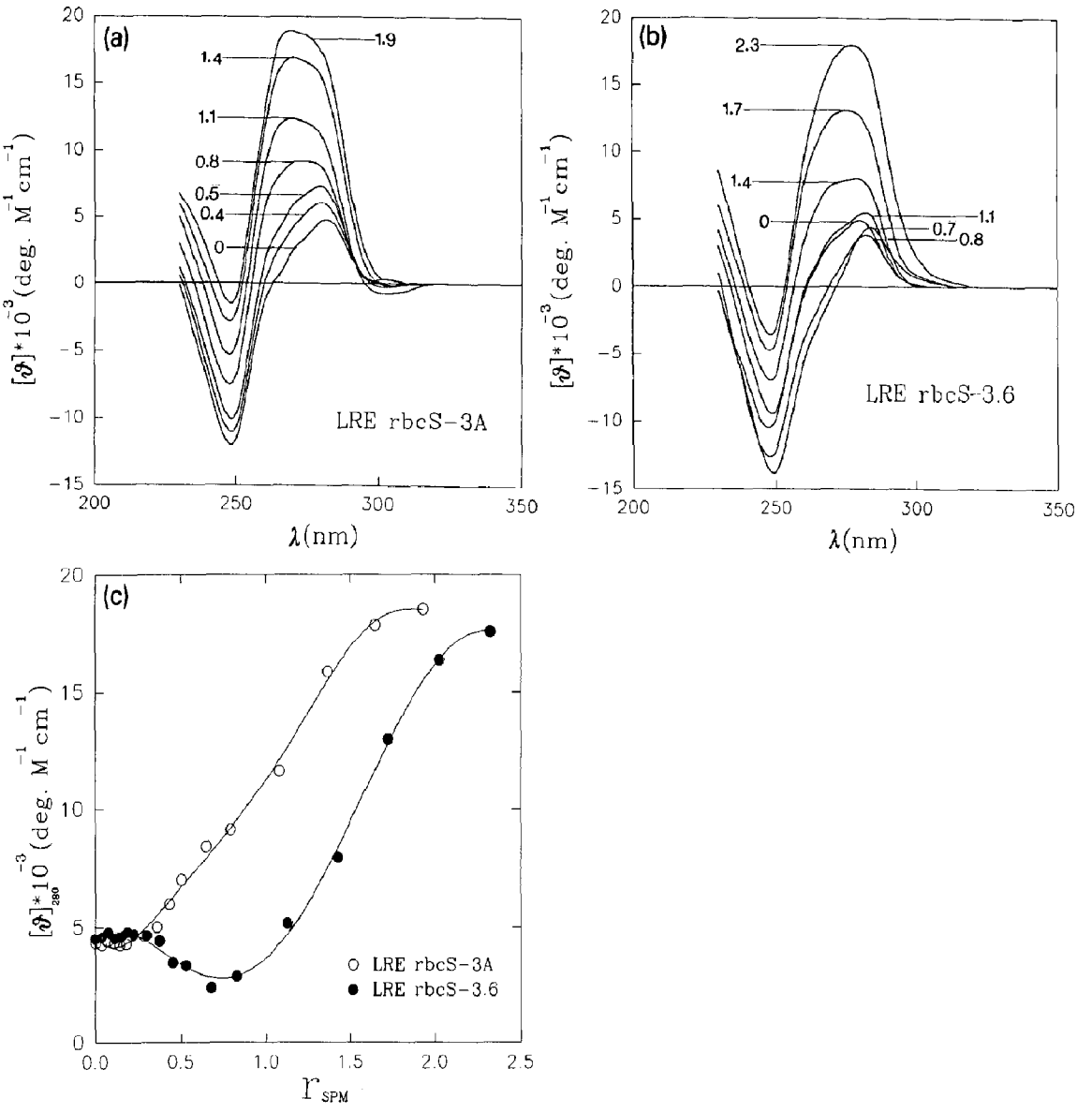

Fig. 5. CD spectra of the complexes between spermine and (a) the LRE of the rbcS-3A gene, and (b) the LRE of the rbcS-3.6 gene in 1 m*M* Tris pH 7.5 at increasing values of r_{SPM}, as an reported in the figures. In (c) the molar ellipticities [ϑ] (at λ = 280 nm) vs. r_{SPM} are reported.

(ii) Decreasing ionic strength, the increase of the band at 280 nm becomes more evident and starts at lower r_{SPM}. (iii) The features of the CD spectra of the complexes between spermine and the LRE of the rbcS-3.6 gene appear more complex. At the lowest and at the highest ionic strength, the variations in CD spectra induced by the polyamine are similar to those of the other LRE, except that in both cases the value of r_{SPM} necessary to induce the CD perturbation is higher. (iv) At the intermediate ionic strength, namely 5 m*M* Tris, the behaviour seems different. In fact the positive band decreases and red-shifts, about 10 nm, while the negative band strongly increases. It is worth noting that the decrease and the red-shift of the positive band, in the case of rbcS-3.6 LRE, seems also to happen at 1 and 10 m*M* Tris,

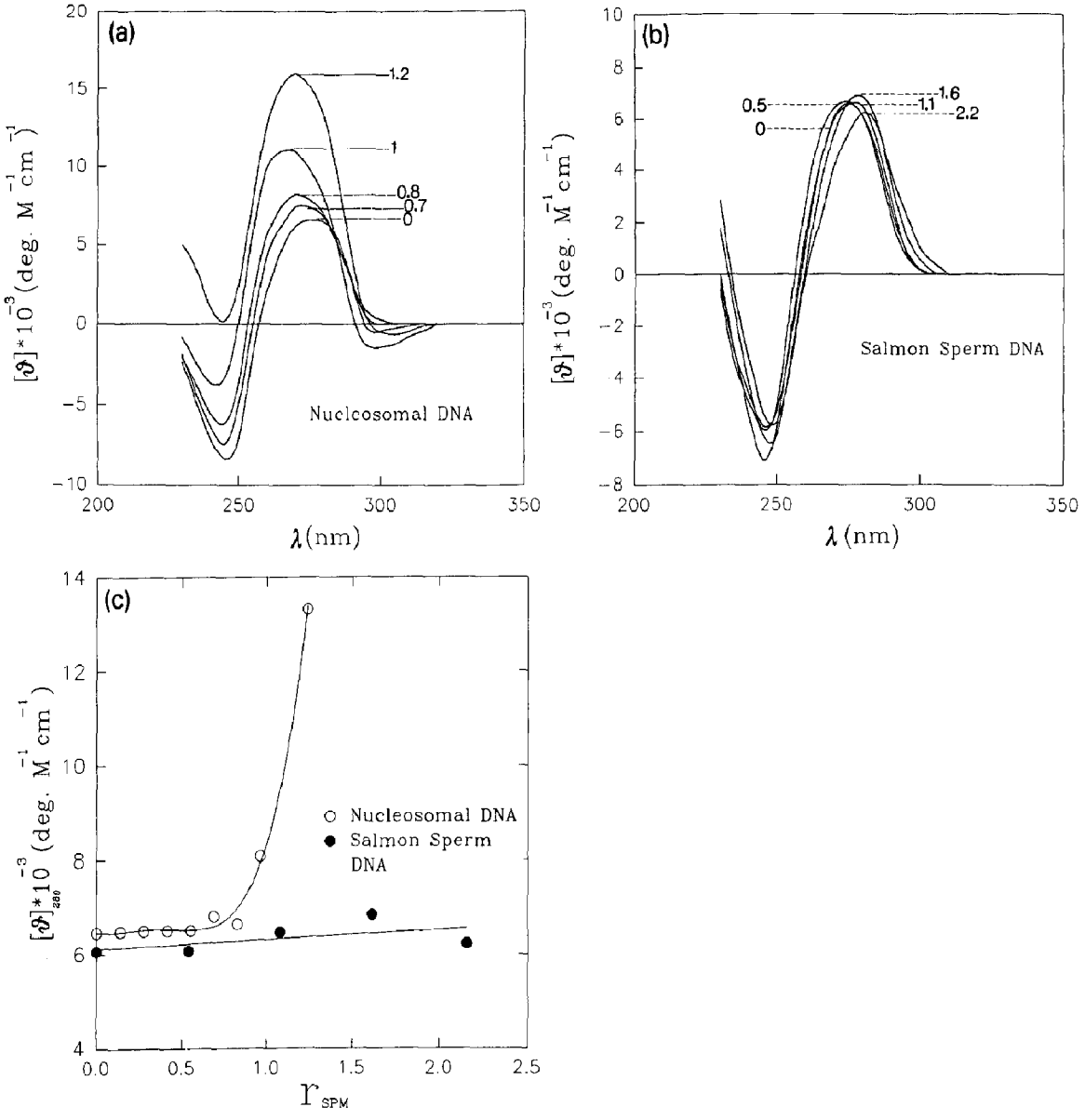

Fig. 6. CD spectra of the complexes between spermine and (a) nucleosomal DNA, and (b) salmon sperm DNA, in 5 m*M* Tris pH 7.5 at increasing values of r_{SPM}, as reported in the figures. In (c) the molar ellipticities $[\vartheta]$ (at $\lambda = 280$ nm) vs. r_{SPM} are reported.

although in a limited range of r_{SPM} values, as a pretransition (Fig. 3(c) and Fig. 5(c)). It must be taken into account that the red-shift of the positive band, at the higher neutralization ratio, could derive from the presence of large complexes aggregates, scattering the radiation: however, also in this respect, it is interesting to point out the different behaviour of the two LREs.

We believe that the variation induced by spermine interactions on the CD spectra of the two LREs cannot be attributed to conformational transition from B- to A-type structure, since from previous results [26] it appears that the polyamine alone is not able to shift B-type DNA conformation to A-type, but the presence of alcohol is required. Also the results of retardation measurements seem to indicate that

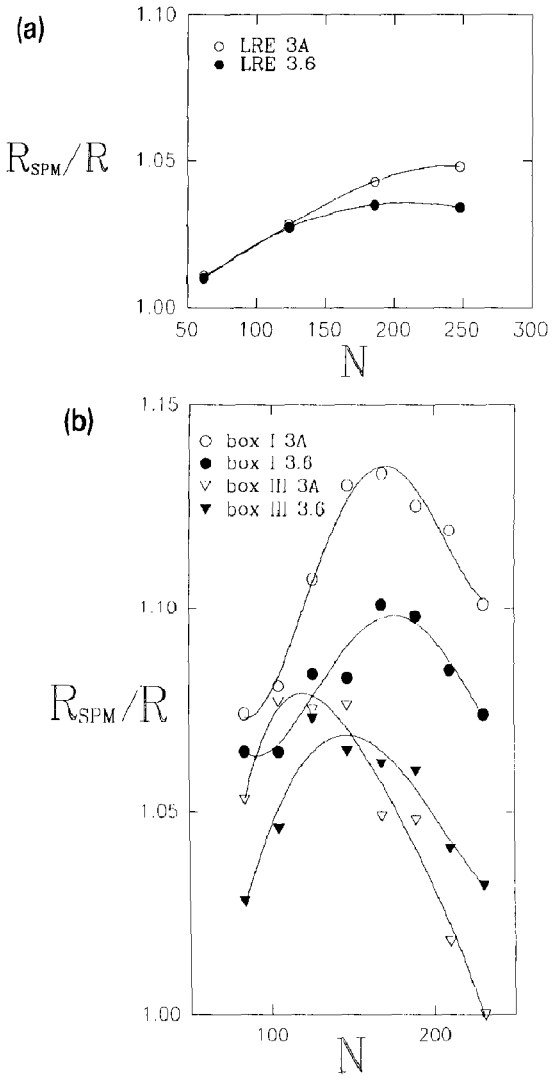

Fig. 7. Ratio between retardation values in presence and absence of spermine (R_{SPM}/R) vs. N. (a) LREs, (b) boxes I and boxes III.

spermine increases DNA twist, while the A-conformation should require the decrease of twist angle. Finally, the CD variations induced by the polyamine depend on DNA molecular weight; this feature renders unlikely a B–A conformational transition.

Taking into consideration previous results obtained in many different laboratories [27,28] showing that polyamines are effective in inducing DNA condensation and interactions between different chains, we suggest that the CD features of the complexes between LREs and spermine could derive from the formation of asymmetric arrangements of complexes molecules in solution. This model is supported, also, by the study of the influence of spermine on CD spectra of nucleosomal and high molecular weight DNA. In fact, while in the case of nucleosomal DNA, the polyamine is able to organize an asymmetric arrangement of complexes molecules, in the case of long DNA chains the ordering of the complexes molecules should be kinetically more difficult. Our hypothesis is, also, in agreement with previous studies by Damashun et al. [29], and Becker et al. [30]. These authors found, using sonicated mixed DNA

sequences, in the presence of spermine, large variations of CD spectra and low angle X ray diffraction patterns, suggesting the presence of ordered molecules aggregates.

Although the structural features of the complexes arrangements cannot be derived from CD measurements, requiring more powerful chemico-physical techniques such as light-scattering or linear dichroism. However, some interesting considerations can be derived from our results on the relationship between spermine binding and DNA bending. At the two ionic strengths (1 mM and 10 mM Tris) where CD features of the two LRE-SPM complexes are similar, the CD spectra variations start at higher r_{SPM} values in the case of rbcS-3.6 LRE, which has the lowest curvature, and the difference in the r_{SPM} values is larger at lowest ionic strength. This behaviour could be easily explained postulating that spermine association constant to the two sequences is influenced by their different superstructural features.

In conclusion, the reported results, both from CD studies and gel electrophoretic measurements, strongly suggest that spermine is able to discriminate between highly homologous DNA sequences according to their curvature and thus could be an excellent candidate as regulator of DNA superstructures in biological systems

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