Sequence-directed curvature of repetitive *Alu I* DNA in constitutive heterochromatin of *Artemia franciscana*

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ABSTRACT

An *Alu I* family of repeated DNA sequence 113 bp in length was found to be the major component of the heterochromatin in *Artemia franciscana*. On the basis of the analysis of cloned oligomeric (monomer to hexamer) heterochromatic fragments we predicted that the sequence could produce a stable curvature in chromosomal DNA. This prediction was confirmed by polyacrylamide gel electrophoresis analysis and by electron microscope observations. The anomalous mobility of these fragments is reversed when the DNA samples are electrophoresed in the presence of distamycin A. Moreover treatment of living *Artemia* with this drug produces visible decondensation of heterochromatic masses in the interphase nuclei.

INTRODUCTION

The genomes of higher eukaryotes contain varying amounts of tandemly repeated DNA sequences (satellite DNA) that are typically located in the constitutive heterochromatin, the most highly condensed region of the interphase chromosome (1-3).

Various functions have been proposed to justify the presence of this repetitive DNA in the cell. For instance the heterochromatic DNA could exert influence on higher-order chromatin condensation at metaphase (4), it could regulate gene expression (5) and it could be involved in spindle attachment during the metaphase (6). However it is clear that whatever the function is, it depends on the molecular structure. In other words, to understand the molecular mechanisms of action of heterochromatin calls for knowledge of the intrinsic DNA conformation as well as for the identification of proteins with the potential role of maintaining the complex order of heterochromatin structure.

A great number of satellite DNAs have been found to have unit repetition ranging between 100-400 bp in length (2) with a higher AT content.

It was recently shown that blocks of three to six consecutive adenine residues arranged in phase with the pitch of the DNA helix are the cause of a sequence directed DNA curvature that could confer a specific secondary structure to the chromosome (7). An example of extremely curved DNA was revealed in kinetoplast minicircular DNA of the trypanosomide protozoa by Marini et al. (8), extensively studied by several physicochemical methods (9-11) and recently also visualized by electron microscopy (12).

Since these observations, numerous other examples of naturally occurring DNA curvature have been reported (13-16).

Recently Radic et al. (17), using the antitumor AT-specific drug distamycin A, have demonstrated

that mouse satellite DNA curvature can play a fundamental role in determining the condensation of the heterochromatin.

We have shown in the brine shrimp *Artemia franciscana* the presence of a DNA family of repeats (*Alu I* family) that is positively correlated with the chromocenters present in the interphasic nuclei and with heterochromatic blocks detectable in the prophase chromocenters (18).

The repetitive heterochromatic DNA, which represents 4-5% of the whole haploid genome in this specie, is medially or scarcely represented in other sibling species and parthenogenetic populations of the genus (19).

The *Alu I* DNA fragment, which shows a clustered organization, has a monomeric unit of 113 bp in length and is characterized by a high AT content (71%).

In this paper we analyze the secondary structure of cloned tandem copies (monomer to hexamer) of a heterochromatic unit. We observed that the *Alu I* DNA fragments contain intrinsic stable curvature which can change as a function of the length of the DNA fragment.

In addition, experiments carried out in the presence of distamycin A have shown that treatment of living nauplii with this drug modifies the condensation of the chromocenters, suggesting an essential significance of DNA curvature in the organization of constitutive heterochromatin.

MATERIALS AND METHODS

Cloning and sequencing of *Alu I* multimeric fragments

The multimeric fragments were obtained by a partial digestion with *Alu I* enzyme of genomic DNA of *A. franciscana* (18).

The fragments corresponding to the monomer, 2-mer, 3-mer, 4-mer, 5-mer and 6-mer were separated on 1.5% agarose gel, eluted and then cloned, according to Maniatis et al., (1982), in the *Sma I* site of pUC18, where the *Hind III* site of the polylinker was replaced by the *Eco RI* site (pUC18m; 21).

Positive clones were identified by colony hybridization using the cloned *Alu I* monomer as probe (19). The fragments were then obtained from the recombinant plasmids by cleavage with *Eco RI* enzyme. They were sequenced by the dideoxy-nucleotide chain-termination method (22) using deoxyadenosine 5' α [35 S] triphosphate, denatured plasmid DNA template (23) and the *E. coli* klenow fragment of DNA polymerase I.

Polyacrylamide gel electrophoresis

The mobility shift electrophoresis of the multimeric fragments was performed on 8% polyacrylamide gel in 40 mM Tris-HCl (pH 7.8); 20 mM Sodium Acetate ; 2 mM EDTA for 15-18 hrs at 2.5 V/cm. according to Marini et al. (8) with recirculation buffer. Gel was analysed by ethidium staining.

Distamycin A was added directly to DNA samples, according to Wu and Crothers (11) to the concentrations indicated in Figure 4.

Electron microscopy

Samples were prepared for EM as described by Griffith et al. (12). The DNA was mounted directly onto very thin carbon film supported by 300 mesh copper grids, on which is placed a 20 μ l drop of the DNA solution containing 1 μ g/ml of DNA fragment, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA; 150 mM NaCl

FIGURE 1 Nucleotide sequence and molecular organization of heterochromatic *Alu I* DNA

Consensus sequence of *Alu I* monomeric fragment: blocks of A residues, position 14-18; 35-38; 54-56; 67-69; 75-78; 87-90; 92-95; 98-101, are represented in bold typeface.

The scheme shows the tandem organization of *Alu I* fragments. N, 2N, 3N, 4N, 5N and 6N represent monomer (113 bp), 2-mer (226 bp), 3-mer (339 bp), 4-mer (452 bp), 5-mer (565 bp) and 6-mer (678 bp) respectively.

and 2 mM Spermidine. The grids were then processed as described by Griffith et al. (12) and stained with uranyl acetate. The electron microscope (JEM.100SX) was operated at 40 KV.

Preparation of nuclei

Cells were obtained from living nauplii. The detailed procedure for the cytological preparation of nuclei has already been described (18). To detect the effect of distamycin A *in vivo*, the cysts were developed in sea water in the presence of 200 ng/ml of the drug.

RESULTS

Cloning and sequencing of *A. franciscana* *Alu I* heterochromatic DNA

We isolated multimeric fragments ranging from 113 to 678 bp in length by partial digestion of *A. franciscana* DNA with *Alu I* (18). We cloned the fragments corresponding to monomer, dimer, trimer, tetramer, pentamer and hexamer of the unit of 113 bp in pUC18 and sequenced them to determine their organization in chromosomal DNA. Figure 1 shows the consensus sequence of 113 bp (19, 24), and reveals that the unit fragments are linked together in a head to tail orientation.

Analysis of monomeric sequence has revealed an AT content of 71%, most of it in homogeneous blocks of 3-5 residues with a symmetrical distribution between the two strands. In particular, 8 stretches of adenine are almost in phase with the helical repeat, with the exception of the block from 92 to 95. These results suggest that the periodic positioning of curvature once per helical turn could force the fragment to curve systematically in a plane. The occurrence of a bend with another periodicity should result in non planarity of the curved DNA (25). If this is true for a single repeated unit, repetition occurrence in the tandem organization of the *Alu I* family of repeats suggests an increasing distortion of the heterochromatic DNA as a function of the number of the monomeric units.

Polyacrylamide gel and electron microscope analysis of the curved heterochromatic DNA

To demonstrate the correlation between the DNA sequence and the curvature of the

FIGURE 2 K-factor values plotted versus monomers number

Anomalous migration of the fragments corresponding to monomer, 2-mer, 3-mer, 5-mer, 6-mer was analyzed on 8% polyacrylamide gel at 4°C and 20°C. K-factors were calculated from the gel at 4°C (o-o) and 20°C (o-o) and plotted versus the number of monomeric units.

Insert shows gel migration at 4°C of 0.1 µg of 6-mer (728 bp); 5-mer (615 bp); 3-mer (389 bp); 2-mer (276 bp) and monomer (163 bp) lanes 1 to 5 respectively. Lane 6 contains 0.5 µg of ΦX 174 DNA digested with Hae III as control of molecular length.

The length of the heterochromatic fragments used for electrophoresis analysis is longer than the theoretical size (see legend of Fig. 1) by the 50 bp belonging to the polylinker of pUC18 plasmid.

heterochromatic DNA, we analyzed the cloned fragments on polyacrylamide gel using the procedure described by Marini et al. (8).

Fragments corresponding to monomer, 2-mer, 3-mer, 5-mer and 6-mer on 8% polyacrylamide gel

FIGURE 3 Visualization of bent hexameric *A/u* DNA fragment

When the cloned 678 bp heterochromatic fragments were mounted as described in Materials and Methods more circular form of different degree of curvature are present (Panel A). When control DNA fragments of the same length are prepared for EM no circles are observed (Panel B).

The grids are mounted at r.t., but the samples are chilled on Ice for 10 minutes and all subsequent steps carried out on ice.

Bar equals 1 μm .

migrate more slowly than the length predicted from the DNA sequence (see insert of Figure 2). Migration of fragments on a control 1% agarose gel is consistent with the real length (data not shown).

Plotting the K-factor (the ratio of the gel electrophoretic mobility of a fragment to that expected of a normal DNA fragment of the same size) versus the number of the monomeric units, we observe (Figure 2) a small increase of the dimer value compared with the monomer value, while there is dramatic variation between dimer and trimer. For longer repeats (3-mer to 6-mer) the value flattens. Figure 2 also shows that K-values are increased when the running temperature of the gel is reduced from 20 to 4°C. This result is in agreement with the observations of others (8, 26) that gel mobility anomalies are more pronounced at lower temperatures.

When the heterochromatic hexamer fragment is examined by electron microscope it appears as fields of molecules of varying degrees of curvature. This could be the result of conformational transitions, which cause a reduction in the natural distortion of the molecule axis. This can be confirmed by visualization of 3-mer heterochromatic fragments which shows a low amount of circular molecules and

FIGURE 4 K-factor values are affected by binding of distamycin A

K-factor of 6-mer fragment (0.1 ng) was calculated in the presence of different amounts of distamycin A by running a 8% polyacrilamide gel at 4°C (o-o) and 20°C (o-o).

about 70% of c-shaped (data not shown). In spite of this more than 60% of the observed hexameric molecules were circular.

As a control, a 680 bp fragment, not homologous to the heterochromatic DNA, cloned in the *Sma* I site of pUC18m and recovered by *EcoRI* digestion, was prepared. When this fragment, which migrates in polyacrilamide gel at the rate appropriate to its size, was examined by EM, no circles were observed and most of the molecules were straight or only slightly bent (Fig. 3).

To verify that the anomalous migration of the *Alu* I DNA fragments was really due to a curvature, we carried out experiments in the presence of distamycin A, shown by Wu and Crothers (11) to abolish the anomalous electrophoretic behaviour of fragments containing bent helices.

Figure 4 summarizes the data obtained from gels run at two different temperatures (20°C and 4°C) with increasing amounts of distamycin A (as described in Figure 4).

When the K-factor was plotted as a function of the number of drug molecules per bp of DNA, at 4°C a ratio of one molecule of distamycin per 200 bp was sufficient to initiate DNA straightening and one drug molecule per 40 bp gave the DNA the same curvature degree as at 20°C; then the DNA distortion was reduced, at the two temperatures, at the same rate and a ratio of 1 to 10 was necessary to obtain the lowest K-factor of 1.2.

Distamycin A effects *in vivo*

In order to correlate the *in vitro* effects of distamycin A on heterochromatic DNA with biological functions, we investigated the effect of the drug on artemia cells.

FIGURE 5 Distamycin A treated nuclei

Interphasic nuclei from nauplii developed in the presence of 200 ng/ml of distamycin A (panel B) and control nuclei from nauplii grown in the absence of the drug (panel A). Heterochromatic masses are revealed by Giemsa staining.

When *artemia* cysts develop into nauplii in the presence of 200 ng/ml of distamycin A (Figure 5b), chromocentric regions of interphase nuclei appear clearly smaller than the 14-15 chromocenters present in the control nuclei in the absence of distamycin (Figure 5a).

This may be indicative of an action of the drug on higher-order heterochromatin organization as was demonstrated for the centromeric heterochromatin of mouse cells (17).

DISCUSSION

The brine shrimp *Artemia* offers some possibilities for understanding the structures that determine the molecular conformation of heterochromatin, and perhaps its role in the cells.

This genus comprises sibling species and parthenogenetic populations that differ widely in their

content of heterochromatin (19). In *A. franciscana* we have demonstrated that part of the DNA organized in heterochromatic masses is a repetitive *Alu I* DNA fragment of 113 bp in length (monomer) particularly AT rich (71%) and having a reiteration frequency of approximately 6×10^5 copies per haploid genome. This repetitive DNA is practically undetectable in all species and populations that do not exhibit heterochromatic staining (19).

Analysis of the sequence of the monomeric fragment predicts a stable, sequence-directed curvature in the plane. This is due to the presence of blocks of residues repeated in phase with the DNA helix, while the stretch of four A with different periodicity (position 92-95) would introduce writhing of the molecule into the space, causing an additional alteration in DNA secondary structure.

In order to study the heterochromatin condensation occurring in artemia cells we cloned and sequenced six heterochromatic *Alu I* DNA fragments ranging from monomer to hexamer in length. The repeated *Alu I* DNA fragments show a tandem organization, so that the addition of one or more identical sequences to the monomer has the effect of increasing the curvature of the molecule as was shown by Koo and Crothers (27) using multimers of synthetic oligomers.

The results shown in Figures 2 and 4 and the electron microscope observations seem to confirm the prevision of a stable sequence specific curvature, with an increasing distortion as a function of the molecular length.

Moreover, a dramatic increase of the K-factor occurring between 2-mer and 3-mer fragments indicates that 3-mer is the DNA fragment length subjected to the highest structural transition, reaching a conformation of a full turn of circle (28). Further the addition to the 3-mer of other heterochromatic units does not significantly change the value of the K-factor. This could indicate that the steric hindrance determined by the molecular structure of the 339 bp heterochromatic fragment does not undergo a great variation even if the length of the DNA fragment is double (6-mer) providing for it a two overlapping circles structure. In fact, circularized hexameric fragments, observed by EM, show a circumference length in agreement with the size of a DNA fragment of about 300 bp.

All these considerations allow us to hypothesize, for artemia heterochromatic DNA, the formation of a solenoid structure. We have also established that the presence of distamycin A largely eliminates the differences in the rates of electrophoretic migration between oligomeric versions of the heterochromatic fragments.

Since it has been demonstrated (11, 29, 30) that the binding of the drug is antagonistic to the curvature, we can assert that the reduction of the K-factor in the presence of distamycin is a consequence of a straightening of the DNA molecules.

In agreement with the *in vitro* experiments, described above, is the cytological data derived from *in vivo* treatment of nauplii with this drug. Interphase nuclei of nauplii treated with distamycin A show incompletely condensed chromocenters when stained with Giemsa.

Similar *in vivo* effects of the distamycin have been observed in many other biological systems (4, 17, 31, 32).

This allows us to suppose that binding of distamycin to heterochromatic DNA affects its ability to

bend and probably its interaction with condensation-specific proteins, with the consequence of a general perturbation of the higher-order organization of heterochromatin.

So far our experiments have not revealed the mechanism by means of which heterochromatin folds. However, the observations that heterochromatic DNA could be made up as a solenoid structure and that heterochromatic DNA condensation is incomplete in the presence of distamycin A *in vivo* seem to indicate an important role for DNA curvature in the heterochromatin structure organization.

We are currently attempting to investigate the mechanism of interaction of a binding protein we revealed and partially purified from *A. franciscana* cells (manuscript in preparation) that specifically binds to heterochromatic DNA.

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