Topoisomerase I action on the heterochromatic DNA from the brine shrimp *Artemia franciscana*: studies *in vivo* and *in vitro*

Daniele CARETTONI, Nicoletta LANDSBERGER, Emanuela ZAGNI, Roberta BENFANTE and Gianfranco BADARACCO*

Dipartimento di Genetica e di Biologia dei Microrganismi, Università di Milano, Via Celoria 26, 20133 Milano, Italy

The genomes of higher eukaryotes contain various amounts of tandem repeated DNA sequences (satellite DNA) typically located in the constitutive heterochromatin, the most highly condensed region of interphase chromosomes. We have previously demonstrated that an *AluI* DNA family of repeats is the major component of constitutive heterochromatin in the brine shrimp *Artemia franciscana*. The analysis of cloned heterochromatic fragments revealed that this repetitive DNA shows a stable curvature conferring a solenoidal geometry to the double helix. In this paper we provide evidence, using the antitumour

drug camptothecin, that, *in vivo*, topoisomerase I cleaves heterochromatin with a frequency comparable with that observed in the whole genome. The analysis of the break sites shows that the enzyme cleaves heterochromatic DNA at specific sites characterized by a degenerate consensus sequence. Moreover the enzyme-mediated breaks have, *in vitro*, a degenerate consensus sequence similar to, but not identical with, the *in vivo* one. Some of these sites are influenced by the DNA flanking the heterochromatic insert, suggesting that structural variations could modify the enzyme specificity.

INTRODUCTION

The DNA–protein complex that underlies chromosome organization may exist during interphase in two distinct conformations, diffused (euchromatin) or condensed (heterochromatin). This reflects different levels of condensation resulting from DNA interaction with chromosomal or other proteins [1,2]. Early experiments [3] provided evidence that condensed chromatin, represented either by coding DNA temporarily and reversibly repressed (facultative heterochromatinization of euchromatin) or by non-coding DNA (constitutive heterochromatic regions), is biologically inactive.

Constitutive heterochromatin is composed mainly of specific structural proteins and of highly repeated DNA, ranging between 10 and 400 bp, arranged in long tandem arrays and with a sequence characteristic of each family of repeats [4].

Recent studies have demonstrated that many repetitive DNAs [5] and, in particular, pericentromeric heterochromatin from mouse [6] and telomeric heterochromatin from the brine shrimp *Artemia franciscana* [7,8] contain an intrinsic curvature determined by the nucleotide sequence. The authors [5–8] suggested that this curvature can confer to DNA a specific structure responsible for heterochromatic condensation.

The brine shrimp *Artemia* offers some possibilities of understanding 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 [13]. The most important component part of the heterochromatic DNA in *A. franciscana* is a repetitive *AluI* DNA fragment of 113 bp in length (monomer) particularly AT-rich (71%) and having a reiteration frequency of approx. 6×10^5 copies per haploid genome [13]. Our recent results [7] have shown that this satellite DNA exhibits a sequence induced curvature conferring a solenoidal geometry to the double helix.

It has been suggested that this particular DNA conformation is the preferential target of topoisomerase I. In fact Caserta et al. [9] and Krogh et al. [10] demonstrated the high preference of binding and cleavage of bent DNA by topoisomerase I *in vitro*,

and we provide evidence in the present paper of direct interactions between topoisomerase I and heterochromatic bent DNA *in vivo*. All this could be important in modulating the topoisomerase events that occur during the physiological modification of chromosomal architecture.

It is well known that the basic role of eukaryotic topoisomerase I is to keep constant the DNA conformation [11,12] through the removal of the torsional strain and of the topological alterations that may accumulate during various genetic processes. This would permit the cyclic repetitions of reactions that progressively modify the DNA topology, as replication and transcription.

In the present paper we demonstrate that topoisomerase I cleaves the *A. franciscana* constitutive heterochromatin with the same frequency of the whole genome and that cleavable-complex sites detected on heterochromatic DNA *in vivo* show a degenerate consensus sequence. Moreover, we characterize *in vitro* the activity of topoisomerase I on the heterochromatic bent DNA.

EXPERIMENTAL

Artemia strain

Artemia franciscana was provided as dry cysts by the Laboratory of Mariculture of the University of Ghent, Ghent, Belgium.

Artemia growth and genomic DNA preparation

Cysts of *A. franciscana* were developed to nauplii in synthetic sea water, as previously described [13], in the absence or in the presence of camptothecin (provided by Sigma) at concentrations specified in the legend of the Figures. Hatched nauplii were collected, washed with 10 mM Tris/HCl/1 mM EDTA, pH 8 (TE buffer) and homogenized in a Potter homogenizer. Nuclei were collected by centrifugation in a Beckman Microfuge at 5000 g for 10 min, resuspended in TE and lysed by addition of SDS at final concentration of 1%. Sodium acetate, pH 8, was added to a final concentration of 0.3 M. After 10 min at room temperature the lysate was deproteinized by phenol extraction. DNA was successively extracted from the water phase and

* To whom correspondence should be sent.

interphase. After treatment for 30 min at 37 °C with proteinase K (100 µg/ml), the DNA was purified with repeated phenol and phenol/chloroform extraction.

Cloning and sequencing of *AluI* multimeric fragments

The multimeric fragments were obtained from *A. franciscana* DNA by partial digestion with *AluI* [14] and cloned as previously described [7] in the *SmaI* site of pUC18, where the *HindIII* site of the polylinker was replaced by the *EcoRI* site (pUC18m) [15]. Positive clones pU-He1⁺ and pU-He6⁺, containing respectively heterochromatic monomer (113 bp) and hexamer (678 bp) fragments, were used in the present study.

The nucleotide sequence of the inserted fragments were carried out by the dideoxynucleotide chain-termination method [16].

Topoisomerase I purification

Topoisomerase I purification to near homogeneity from *A. franciscana* nuclei has been described previously [17].

Identification of the cleavable-complex sites *in vivo*

The identification of the cleavable-complex sites was obtained by the analysis of the covalent interaction between topoisomerase I and DNA in the presence of camptothecin and is realized following, with minor modifications, the protocol described by Porter and Champoux [18]. Genomic DNA (2 µg) from *Artemia* grown with or without camptothecin, prepared as previously described, was denatured by adding NaOH to final concentration of 250 mM.

After 5 min at room temperature the sample was neutralized by adding ammonium acetate, pH 4.6, to 0.2 M and precipitated with 2 vol. of cold ethanol. The DNA pellet was then resuspended in 10 µl of annealing buffer [40 mM Tris/HCl (pH 7.5)/10 mM MgCl₂/50 mM NaCl/2 mM dithiothreitol], 6 ng of primers consisting of synthetic oligonucleotides 33 or 40 bases long, respectively complementary to nucleotides 80–113 of the left strand or 73–113 of the right strand of the monomeric *AluI* sequence shown in Figure 3 (below) and incubated 15 min at 37 °C. Then 1 µl of a mixture containing dGTP, dCTP and dTTP at a concentration of 1.5 mM each, 1 µl of [α -³²P]dATP (10 µCi) and 3 units of T7 DNA polymerase (provided by Promega) were added and incubated for 5 min at room temperature. Afterwards, 7 µl of a mixture of dGTP, dCTP and dTTP at a concentration of 100 µM each and of dATP (70 µM) were added and left for a further 5 min at 37 °C. The reaction was stopped with 6 µl of 95% formamide/0.01% Bromophenol Blue/0.01% xylene/cyanol. The replication of the template DNA was evaluated by cold trichloroacetic acid precipitation and liquid-scintillation counting of incorporated ³²P radioactivity.

The replicated DNA was denatured at 85 °C for 2 min and analysed on a 6%-polyacrylamide sequencing gel as described by Sambrook et al. [19].

Identification of the cleavable-complex sites *in vitro*

Reaction was carried out in the presence of 25 mM Tris/HCl (pH 8.0)/0.1 M NaCl/0.5 mM EDTA, 40 ng of covalently closed pU-He6⁺ or *PvuII*-restricted plasmid and 80 units of topoisomerase I. After 5 min at 30 °C, 50 µg/ml camptothecin was added and incubated for another 5 min. The reaction was stopped by addition of SDS to 1% and of 100 µg/ml proteinase K. The samples were incubated for 30 min at 37 °C and then deproteinized by phenol and chloroform extractions. The DNA

contained in the water phase was treated as described above, except for the utilized primers (pUC/M13 forward and reverse primers from Promega).

Analysis of the camptothecin-treated DNA on alkaline sucrose gradients

The camptothecin-untreated and -treated DNA was denatured with 0.1 M NaOH and incubated at 65 °C for 30 min. The samples were then layered on to a linear 10–30% (w/w) sucrose gradient made up in 0.1 M NaOH. Centrifugation was carried out in a Beckman 55 Ti rotor for 180 min at 250 000 *g* (20 °C). The gradients were fractionated from the bottom and analysed on 1%-agarose gels in TBE 1X buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8). Standard DNAs (λ = 48 502 bp; ϕ X174/*AvaI* = 5386 bp) were centrifuged and fractionated under the same conditions to evaluate the genomic DNA size of the corresponding fractions.

RESULTS

Effect of camptothecin on the embryonic development of *Artemia*

The fertilized *Artemia* eggs develop to swimming larvae inside the female ovisac. During development, depending on environmental conditions, gastrula can continue directly to the nauplius stage or stop in a cryptobiotic status creating a shell that permits a permanent maintenance of the dehydrated embryo as 'cyst'.

Figure 1 Effect of camptothecin on *Artemia* development

Artemia cysts were hydrated in the absence (▲) or presence of 5 (●) and 50 (□) µg/ml of camptothecin. The hatching (inset in a) and nauplius (inset in b) embryonic stages were directly observed and counted under the optical microscope, the ratio of the hatching larvae (a) and of viable nauplii (b) in comparison with the number of cysts utilized in each experiment (about 250–300) being estimated. Dimethyl sulphoxide (2% final concn.) effect is plotted on the ○ graph.

Figure 2 Topoisomerase I induces breaks on genomic DNA

DNA purified from *Artemia* grown in the absence (i) or presence [20 (ii) and 50 (iii) $\mu\text{g/ml}$] of camptothecin was analysed by alkaline sucrose gradient as described in the Experimental section. (a) Shows the distribution of fractionated genomic DNA on 1% agarose gel. The distribution of heterochromatic *AluI* DNA (b) was revealed by Southern blot of the gel in (a). The filter was hybridized with ^{32}P -labelled 113 bp *AluI* fragment.

(for a review, see [20]). To study *in vivo* topoisomerase I activity on the heterochromatic *AluI* DNA family [13] we analysed the developing embryos to see whether they are permeable to the enzyme-specific inhibitor camptothecin. Figure 1 represents the time course of *Artemia* development in the absence or presence of the drug. The results show that, under our experimental conditions (see the Experimental section), *Artemia* cysts require about 26–28 h for hatching (inset to Figure 1a), while they need 35–40 h to generate a swimming nauplius larva (inset to Figure 1b).

When the artemia cysts are hydrated in the presence of camptothecin (see the legend to Figure 1), embryonic development reaches hatching at the same time as the control, but the embryos remain inside the hatching membrane for many hours and only few transform into swimming nauplii (see the accumulation of the hatching larvae in Figure 1a and the small number of nauplii in Figure 1b). However, the new nauplii grown in the presence of camptothecin cannot proceed to development, but die at this stage. Dimethyl sulphoxide, the camptothecin solvent, does not influence embryonic development (Figure 1).

Constitutive heterochromatin is a target *in vivo* of topoisomerase I

Eukaryotic topoisomerase I forms a cleavable complex with duplex DNA in which the enzyme is covalently attached to a 3' phospho group at the site of an enzyme-induced single-strand nick. Addition of camptothecin traps the normally transient cleavable complex and results in single-strand DNA cleavage [21].

We purified the DNA from hatched *Artemia* developed in the absence or presence of camptothecin (20 or 50 $\mu\text{g/ml}$). The samples were fractionated on alkaline linear sucrose gradients and analysed by agarose-gel electrophoresis (Figure 2). In a

parallel sucrose gradient, λ DNA (48 502 bp) and $\phi\text{X174}/\text{AvaI}$ DNA (5386 bp), utilized as size markers, sediment respectively into fractions 9–11 (well 5) and 19–21 (well 11; results not shown). Afterwards the DNA was transferred and hybridized with the cloned *AluI* monomer as probe (Figure 2b). It appears evident (Figure 2a) that the genomic DNA is frequently cleaved by topoisomerase I. In fact, in the presence of camptothecin, a great amount of DNA is localized on the top of the gradient (smaller than ϕX174 DNA), while in the absence of the drug the DNA has a higher density at about the λ DNA position).

Figure 2(b) shows that heterochromatic DNA is also frequently cleaved by topoisomerase I, since its distribution is similar to the genomic one. Moreover, the heterochromatic DNA treated with camptothecin shows two broad bands in the central part of the gradient. One may speculate that heterochromatic DNA exists in two different conformations, one more accessible to the topoisomerase I action than the other one. This could determine, in the presence of camptothecin, two different populations of *AluI* DNA fragments. The sucrose gradient separates these two populations as distinct peaks with overlapping tails, discriminated as two broad bands on agarose-gel electrophoresis.

To verify that the different transference conditions experienced by the DNA fragments with higher M_r did not invalidate this result, the same filter was probed with genomic DNA purified from an artemia population virtually devoid of *AluI* heterochromatic DNA (*Artemia parthenogenetica* from Tsing-Tao Bay) [13]. The experiment (results not shown) confirmed the result described above.

To determine whether topoisomerase I recognizes specific sites *in vivo* on the heterochromatic DNA, we analysed the position of the cleavable complexes using the primer-extension method described in the Experimental section. Figure 3 shows the presence, *in vivo*, of specific cleavage sites at nucleotides 11, 45, 73, 85 and 113 on the right strand and at nucleotides 10, 42, 93 and 113 on the left strand. Furthermore, analysing the intensity

Figure 3 Mapping of the cleavable-complex sites determined *in vivo* by topoisomerase I

Genomic DNA of *A. franciscana* grown in the absence or presence of camptothecin was prepared as described in the Experimental section. Lanes 5 and 6, 4 and 7, 3 and 8, 2 and 9, and 1 and 10 contain the genomic DNA from artemia treated respectively with 0, 5, 10, 20 and 50 $\mu\text{g}/\text{ml}$ of camptothecin. The positions of the cleavable-complex sites were evaluated aligning the reaction products with a primer-extension reaction carried out in the presence of dideoxynucleotides made up on camptothecin-untreated genomic DNA (lanes CATG on the left and on the right side). The *AluI* monomer sequence and cleavable-complex sites are indicated in the middle of the figure.

of the autoradiographic signals of cleavable sites as a function of the amount of camptothecin (Figure 3), we verified that, although the inhibitor does not alter significantly the enzyme sequence specificity, there were three kinds of cleavage sites: (i) strong sites at positions 10 and 42 were detectable when the reaction was stopped with detergents (see the Experimental section), even in the absence of camptothecin, (ii) sites at positions 11 and 85 revealed only in the presence of the inhibitor, even if they do not show any dose-dependence under our experimental conditions, and (iii) finally sites 45, 73, 93, 113 are clearly dose-dependent. By aligning the sequences of the cleavage sites it is possible to identify the degenerate consensus sequence shown in Table 1.

Identification of cleavable-complex sites on heterochromatic DNA *in vitro*

After demonstrating the presence of topoisomerase I cleavage

sites on the heterochromatic DNA *in vivo*, we also investigated the activity of the enzyme on the *AluI* repetitive DNA *in vitro*.

The sequence preference of the cleavage reaction was analysed at the nucleotide level (see the Experimental section) using plasmids carrying a monomeric heterochromatic unit (pU-He1⁺) [7] or an hexameric one (pU-He6⁺).

The experiment reported in Figure 4 shows the cleavage pattern produced, in the absence or presence of camptothecin, with linear and covalently closed pU-He6⁺ plasmids. Five preferential sites at nucleotides 11, 28, 50, 72 and 111 on the left strand and one at nucleotide 108 on the right strand are evidenced. These sites can be mapped, under our experimental conditions, only in the presence of camptothecin, which causes a great accumulation of cleavable complexes [22,23]. The preferential sites at nucleotides 11, 28 and 50 are evidenced in all the repetition units of the heterochromatic hexamer examined. The cleavage sites at nucleotides 72 and 111 on the left strand are

Table 1 *In vivo* and *in vitro* topoisomerase I break sites and consensus sequences

Nucleotide sequences surrounding the 3'P cleavage site (position -1) of topoisomerase I are indicated from the position -6 to the position +5 for both strands *in vitro* (left side) and *in vivo* (right side). The consensus sequences obtained are indicated.

<i>In vivo</i> and <i>in vitro</i> topoisomerase break site			
<i>In vitro</i>		<i>In vivo</i>

5' T A T G T T	3' G G A A A	5' T A T G T T	3' G A A A A
G A T A T T	A G A C T	T G A T T G	C A A A T
G C A C A T	G G T A A	A A G G C T	C T T T T
A A G G C T	C T T T T	C T A T T T	C A A A A
G G T A A T	A G G G G	T A A T A G	C T A C G
3' H O	5' T	3' H O	5' T
G A T G C	A T A C A A	A A A G C	T C C C A T
		T T T C C	G A G A A A
		T T T A C	T T C T A T
		T T A T C	G A T G C A
Consensus sequence			
<i>In vitro</i>		<i>In vivo</i>	
5' G A X X A T	3' X G A X X 3'	5' A A X X A G	3' C X A A A 3'
-6 -5 -4 -3 -2 -1	+1 +2 +3 +4 +5	-6 -5 -4 -3 -2 -1	+1 +2 +3 +4 +5

recognized only on monomer A, while the site at nucleotide 108 is cleaved only in monomers C-E on the right strand, but not in the F monomer. The plasmid topology does not seem to have any effect on the cleavage sites of the heterochromatic DNA (Figure 4).

Some extra bands visible in the absence of topoisomerase I and camptothecin (see lanes 5 and 8) are due to T7 DNA polymerase 'pauses' on supercoiled plasmid; in fact these bands are also present when the sequencing reaction is carried out on a covalently closed plasmid, while they are absent when a linear plasmid was used (see lanes 6 and 7).

When the same experiment was carried out using the plasmid with the monomeric insert (pU-He1⁺), sites at nucleotides 11, 28, 50 and 111 on the left strand and 108 on the right one were still present, whereas the site at nucleotide 72 was lost, suggesting that its presence depends on the length of heterochromatic insert (results not shown). Moreover, we observed that a reduction of the heterochromatic DNA curvature, obtained when the incubation temperature was increased [11], does not change the cleavable-complex pattern (results not shown).

Experiments carried out utilizing linear end-labelled DNA followed by topoisomerase I cleavage [10] confirmed the validity of the primer-extension technique (results not shown).

Also, *in vitro*, the analysis of the cleavage sites reveals the presence of a degenerated consensus sequence (see Table 1) similar to, but not identical with, the *in vivo* consensus sequence.

DISCUSSION

It is well known that the characteristic conformation of the heterochromatic DNA and its modulation during the cell cycle

and cell differentiation could participate in determining chromosomal architecture. Since it has been suggested that topoisomerase I has a basic role in various genetic processes such as replication, transcription, chromosomal condensation and decondensation, we investigated whether bent DNA in *A. franciscana* is a target, *in vivo*, of topoisomerase I.

Our experiments demonstrate that the specific topoisomerase I inhibitor camptothecin arrests the embryonic development of *Artemia* at the hatching stage. This can be understood with the knowledge that development between hydration and hatching takes place in the absence of DNA synthesis and cell division. In fact all processes involved in development from gastrula to nauplius utilize the pre-existing cells (3000-4000 per gastrula), and most DNA and RNA synthesis occurs after hatching, as demonstrated by Morris [24] and Pierandrei-Amaldi et al. [25]. All this, and the knowledge that replication and transcription depend on topoisomerase I activity [26], could explain why camptothecin shows its activity only from the hatching stage.

Our *in vivo* experiments, carried out in the presence of camptothecin, demonstrate that the heterochromatic *AluI* DNA is frequently cleaved, indicating that, in spite of its compaction, it can be accessible to topoisomerase I. However, it is our intention to understand the true conformation of the topoisomerase I target DNA: highly condensed with proper heterochromatic factors or locally decondensed?

The *in vivo* mapping of the cleavable sites reveals the presence, on heterochromatic *AluI* DNA, of specific sites recognized by the enzyme and that the repetitive DNA is frequently nicked as well as the remaining genomic DNA (see Figures 2 and 3).

This is confirmed by our *in vitro* experiments that reveal the presence of cleavage sites on heterochromatic DNA. When an oligomeric (6-mer) heterochromatic fragment was used, the sites 11, 28 and 50 were recognized on each monomeric unit, while those localized at nucleotides 72 and 111 and at nucleotide 108 in the complementary strand appear or disappear respectively in the monomer closest to the boundary between the heterochromatic DNA and the plasmid DNA.

This observation seems to indicate that flanking DNA modifies the preferential sites recognized by topoisomerase I, probably by inducing structural variation in heterochromatic DNA close to the boundary with the plasmid. In agreement with this, only the constitutive sites observed on the hexameric fragment are maintained using a monomeric unit.

The results regarding the cleavage sites and the recognized consensus sequence obtained *in vitro* reveal that topoisomerase I covalently binds a T residue and shows a high preference for an A or a T at position -2. This greatly accords with the consensus sequences found by other groups [18,21,27]. The *in vivo* experiments indicate that the enzyme binds a G or a T residue, recognizing an A/T in position -2, a C in +1 and an A/T-rich region from +3 to +5. It is possible to explain the differences existing between the *in vivo* and *in vitro* cleavage sites and their respective consensus sequences, knowing that topoisomerase I specificity depends on DNA structure [10,27]. In fact *in vivo* the interactions with structural proteins could modify the intrinsic curvature of the heterochromatic bent DNA and could shield some recognition sites from topoisomerase I catalytic activity. On the other hand, *in vitro*, the heterochromatic DNA is devoid of proteins, which results in total accessibility to topoisomerase I cleavage.

Although heterochromatin comprises a large portion of the genome in many eukaryotes, it contains few known genetic functions. However, one well-documented phenomenon, 'position effect variegation' has been extensively studied in the fruitfly *Drosophila* (for a review see [28]). Cytological observations of

Figure 4 Mapping of the cleavable-complex sites determined *in vitro* by topoisomerase I

Cleavable-complex sites were evidenced on heterochromatic DNA sequence as described in the Experimental section. Supercoiled pU-He6⁺ plasmid (Sc) or *Pvu*II-restricted plasmid (L) containing the heterochromatic *Alu*I hexamer were used as substrates for 80 units of topoisomerase I (lanes 1–4 and 9–12). Camptothecin (50 µg/ml) was present in reactions 1, 3, 10 and 12. Positions of cleavable-complex sites on monomeric units (A–F) of the hexameric insert were evaluated by aligning the reaction products with the sequence of both strands of the heterochromatic insert performed with dideoxy chain-termination method (lanes CATG on the left and the right side). Asterisks indicate the heterochromatic units that are not readable on this sequencing gel.

normally active genes placed next to inactive heterochromatin domains suggested that heterochromatin spreads into normally active genes, leading to their repression. It is evident that the formation of transcriptionally inactive heterochromatin requires not only structural proteins, but also enzymic activities such as topoisomerases. It will be important to demonstrate whether topoisomerase I action on heterochromatic DNA is involved in gene activation or repression by assisting in condensing or decondensing whole domains of the chromatin fibre.

This work was supported by the National Research Council of Italy, Special Projects 'Raisa', Sub-project N.4 and 'Ingegneria Genetica' (N. 91.00030. PF 99). N. L. was supported by a fellowship from the Fondazione Anna Villa Rusconi.

REFERENCES

- 1 Hennig, W. (1986) in Results and Problems in Cell Differentiation (Hennig, W., ed.), vol. 13, pp. 176–192, Springer, Berlin, Heidelberg and New York
- 2 Weith, A. (1985) *Chromosoma* (Berlin) **91**, 287–296
- 3 Ris, H. and Kornberg, R. D. (1979) in Structure and Replication of Genetic Material (Prescott, D. M. and Goldstein, L., eds.), pp. 268–361, Academic Press, New York
- 4 Singer, M. F. (1982) *Int. Rev. Cytol.* **67**, 67–112
- 5 Martinez-Balbas, A., Rodriguez-Campos, A., Garcia-Ramirez, M., Sainz, J., Carrera, P., Aymami, J. and Azorin, F. (1990) *Biochemistry* **29**, 2342–2348
- 6 Radic, M. Z., Lundgren, K. and Hamkalo, B. A. (1987) *Cell* **50**, 1101–1108
- 7 Benfante, R., Landsberger, N., Tubiello, G. and Badaracco, G. (1989) *Nucleic Acids Res.* **17**, 8273–8282
- 8 Benfante, R., Landsberger, N., Maiorano, D. and Badaracco, G. (1990) *Gene* **94**, 217–222

-
- 9 Caserta, M., Amadei, A., Di Mauro, E. and Camilloni, G. (1989) *Nucleic Acids Res.* **17**, 8463–8474
 - 10 Krogh, S., Mortensen, U. H., Westergaard, O. and Bonven, B. J. (1991) *Nucleic Acids Res.* **19**, 1235–1241
 - 11 Camilloni, G., Di Martino, E., Caserta, M. and Di Mauro, E. (1988) *Nucleic Acids Res.* **16**, 7071–7085
 - 12 Camilloni, G., Di Martino, E., Di Mauro, E. and Caserta, M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3080–3084
 - 13 Badaracco, G., Baratelli, L., Ginelli, E., Meneveri, R., Plevani, P., Valsasnini, P. and Barigozzi, C. (1987) *Chromosoma (Berlin)* **95**, 71–75
 - 14 Barigozzi, C., Badaracco, G., Plevani, P., Baratelli, L., Profeta, S., Ginelli, E. and Meneveri, R. (1984) *Chromosoma (Berlin)* **90**, 332–337
 - 15 Venditti, S., Caserta, M., Di Mauro, E. and Camilloni, G. (1988) *Biochim. Biophys. Acta* **951**, 139–148
 - 16 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
 - 17 Badaracco, G., Landsberger, N. and Benfante, R. (1991) *Biochem. J.* **282**, 249–254
 - 18 Porter, S. E. and Champoux, J. J. (1989) *Mol. Cell Biol.* **9**, 541–550
 - 19 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
 - 20 Clegg, J. S. and Conte, F. P. (1980) in *The Brine Shrimp Artemia* (Persoone, G., Sorgeloos, P., Roels, O. and Jasper, E., eds.), vol. 2, pp. 11–54, Universa Press, Wetteren, Belgium
 - 21 Porter, S. E. and Champoux, J. J. (1989) *Nucleic Acids Res.* **17**, 8521–8532
 - 22 Hsiang, Y. H., Hertzberg, R., Hecht, S. and Liu, L. F. (1985) *J. Biol. Chem.* **260**, 14873–14878
 - 23 Bendixen, C., Thomsen, B., Alsner, J. and Westergaard, O. (1990) *Biochemistry* **29**, 5613–5619
 - 24 Morris, J. E. (1971) *Comp. Biochem. Physiol.* **39A**, 843–857
 - 25 Pierandrei-Amaldi, P., Felicetti, L. and Campioni, N. (1977) *Dev. Biol.* **59**, 49–61
 - 26 Liu, L. F. and Wang, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7024–7027
 - 27 Perez-Stable, C., Shen, C. C. and Shen, C. K. J. (1988) *Nucleic Acids Res.* **16**, 7975–7993
 - 28 Eissenberg, J. C. (1989) *BioEssays* **11**, 14–17