Nucleic Acids Research, Vol. 19, No. 22 6249–6254

Identification of two homologous mitochondrial DNA sequences, which bind strongly and specifically to a mitochondrial protein of *Paracentrotus lividus*

Marina Roberti, Anna Mustich, Maria N.Gadaleta and Palmiro Cantatore
Dipartimento di Biochimica e Biologia Molecolare, Universita' di Bari, Via Amendola 165/a, 70126 Bari, Italy

Received August 6, 1991; Revised and Accepted October 16, 1991

ABSTRACT

Using a combination of band shift and DNaseI protection experiments, two *Paracentrotus lividus* mitochondrial sequences, able to bind tightly and selectively to a mitochondrial protein from sea urchin embryos, have been found. The two sequences, which compete with each other for binding to the protein, are located in two genome regions which are thought to contain regulatory signals for mitochondrial replication and transcription. A computer analysis suggests that the sequence TTTTRTANNTCYYATCAYA, common to the two binding regions, is the minimal recognition signal for the binding to the protein. We discuss the hypothesis that the protein binding capacity of these two sequences is involved in the control of sea urchin mtDNA replication during developmental stages.

INTRODUCTION

Recent studies (1–4) have shown that sea urchin mitochondrial (mt) DNA replication and transcription occur using mechanisms which are different from those operating in mitochondria of other organisms, such as mammals (5,6). As far as the mt DNA replication is concerned, the data reported by Jacobs et al. (4) in the sea urchin *Strongylocentrotus purpuratus*, support the presence of a short displacement loop in the main non-coding region (between $tRNA^{Thr}$ and $tRNA^{Pro}$). This sequence contains one mtDNA replication origin. The second origin has not yet been identified; it has been suggested that second strand synthesis could initiate from multiple points (7), one of which appears to map upstream the cytochrome b gene (8). MtDNA transcription in sea urchin has been studied using mainly *in vivo* approaches (2, 3). The available evidences suggest that mtRNA synthesis occurs via multiple and overlapping transcription units and that the transcription of ribosomal and messenger genes takes place via mutually exclusive synthetic pathways. Using different criteria two of the multiple transcription initiation sites were located (8, 9), the first in the main non-coding region, probably in the conserved TATATATAA sequence, the second about 500 nt upstream the cyt b gene.

To extend our knowledge of mt replication and transcription in sea urchin, we are developing various experimental strategies. One of these consists in the search for DNA sequences whose binding to mt proteins, could regulate the two processes. Here we report the identification of two homologous DNA sequences in the mtDNA of *Paracentrotus lividus* which are both able to bind strongly and selectively to an mt protein. The data suggest that these sequences, which are located one in the main non-coding region and the other upstream the cyt b gene, bind to the same protein and that they are regulatory signals for mtDNA replication or transcription.

MATERIALS AND METHODS

Preparation of the mitochondrial extract

Mitochondria were prepared from sea urchin blastulae following the procedure described by Cantatore et al. (10). They were then quickly frozen in liquid nitrogen and stored at −70°C. Mitochondria corresponding to 60–100 mg of proteins were thawed on ice, washed once with 100 ml of washing buffer [25 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20% glycerol, 0.25 M sucrose and 0.1 μg/ml of each of the following protease inhibitors: antipain, chymostatin, elastatinal, leupeptin and pepstatin (ACELP)], and centrifuged in the JA-20 rotor of the Beckman centrifuge J2–21 at 10,000 rpm for 15 min. The pellet was suspended in lysis buffer (the same as the washing buffer, except that the sucrose was omitted) at a protein concentration of 10 mg/ml. The mitochondrial suspension was homogenized with five up and down strokes in a glass homogenizer with a tight fitting motor-driven Teflon pestle and lysed with 1% Nonidet P-40 in the presence of 350 mM KCl. The mixture was vortexed and allowed to stand on ice 5 to 10 min. The homogenization was repeated with ten up and down strokes. The mitochondrial lysate was spun at 130,000 g for two hr. The supernatant (S-130) was diluted with the lysis buffer up to 250 mM KCl and applied at a flow rate of 10 ml/h onto a 1.5×3 cm DEAE-Sephacel column (Pharmacia, Inc.) equilibrated with Buffer A (10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 7.5% glycerol, 0.2 mM PMSF, 0.1 μg/ml ACELP) containing 250 mM KCl. The DEAE-Sephacel flow-through was diluted with Buffer A to 175 mM KCl and applied at 8 ml/h onto a 1×2.5 cm Heparine-Sepharose CL-6B column (Pharmacia, Inc.), pre-equilibrated with Buffer A containing 175 mM KCl. The column was washed at

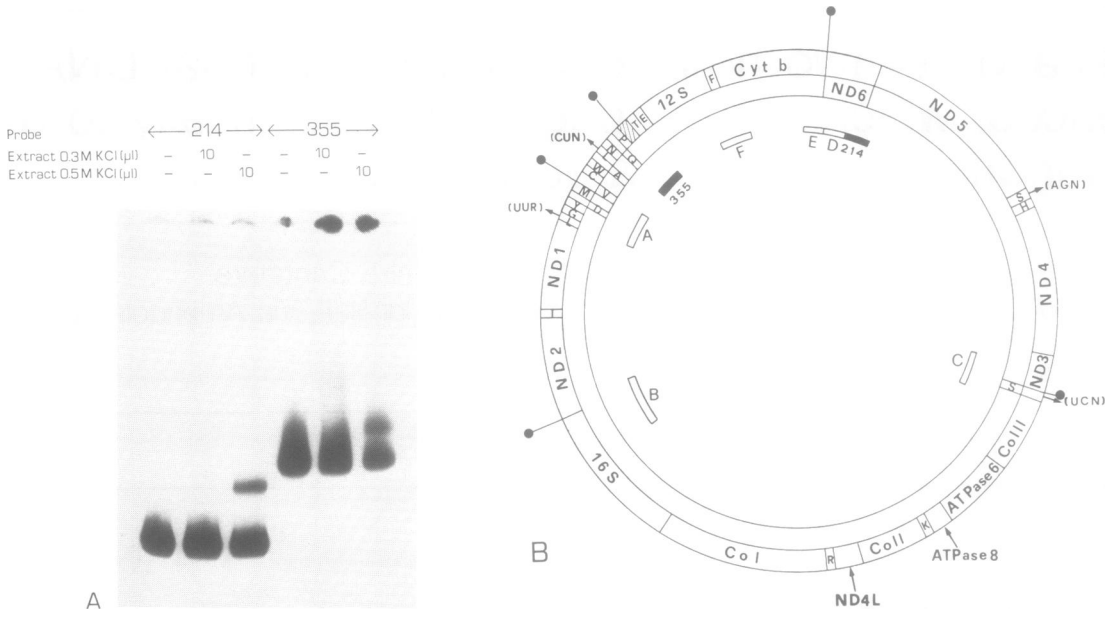

Figure 1. **A**: DNA-binding activity of the protein fractions eluted from the Heparine-Sepharose column. Two absorbance peaks were obtained from the column eluted with 0.3 and 0.5 M KCl; no proteins were eluted with 0.8 M KCl. *P. lividus* mtDNA fragments were 3'end labelled with α^{32}P. incubated with 1 µg of proteins eluted from the Heparine-Sepharose column and electrophoresed in a non-denaturing gel as described in the text. Fragment 355 is a HhaI fragment (positions 1052–1404); fragment 214 is a DdeI fragment (positions 13924–14137). Binding and electrophoresis conditions are described in the text. **B**: Position of the fragments used as probes in the binding to *P. lividus* mt proteins. The coordinates of the fragments on the genome (1) are the following: A, 1754–2245; B, 4209–4660; C, 10120–10483; D, 14138–14299; E, 14307–14593; F, 15496–244. Dark boxes indicate the two fragments that bind to the mt protein extract; dark circles indicate the TATATATAA sequences.

20 ml/h with about 10 vol. of the same buffer and proteins were eluted by successive additions of about 6 ml of Buffer A containing 0.3, 0.5 and 0.8 M KCl. Fractions (about 500 µl) were adjusted to a glycerol concentration of 25%, frozen in liquid nitrogen and stored at −70°C. Protein concentration was determined by the Bradford method (11).

Preparation of DNA probes

DNA probes were obtained by digesting, with the appropriate restriction enzymes, plasmid vectors containing *P. lividus* mtDNA fragments (1,2). After electroelution DNA fragments were end labelled with either Klenow DNA polymerase and [α-^{32}P]dATP or with polynucleotide kinase and [τ-^{32}P]ATP according to standard methods (12).

Gel electrophoresis analysis of DNA binding

Proteins (2–10 µl corresponding to 0.2–1 µg) were incubated with 30,000–50,000 cpm (approximately 0.08 pmol) of end labelled double stranded DNA fragment in the presence of 4 µg of poly (dI-dC). poly (dI-dC) (Pharmacia, Inc.) in a final volume of 20 ul. Incubations were carried out at 25°C for 30 min in 12.5% glycerol, 12 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$, 0.5 mM EDTA, 15 mM DTT, 250 mM KCl, 100 µg/ml RNase-free bovine serum albumine (Pharmacia, Inc.). The samples were layered onto 0.2×16 cm 5% acrylamide gels (bisacrylamide was 0.15%). The gels were electrophoresed in 6.5 mM Tris-HCl pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA at 30 mA at 4°C, until bromophenol blue had reached the bottom of the gel. Gels were prerun for 1 h at 20 mA and buffer was recirculated during both prerunning and sample electrophoresis. At the end of the run gels were dried and autoradiographed.

DNaseI footprinting

Protein samples were incubated with end labelled DNA probes under the conditions described above in a final volume of 90 µl. Then they were treated with 50–100 µg/ml of DNaseI (Boehringer) for 60 sec at 25°C. The digestion was stopped by addition of EDTA to a final concentration of 12 mM; the tubes were placed on ice and their contents were quickly loaded onto non-denaturing gels and run as described above. After autoradiography of the wet gel, the gel slices corresponding to bound and unbound DNA were excised and the DNA was eluted by shaking overnight at 37°C in 0.5 M ammonium acetate, 0.1% SDS, 0.1 mM EDTA, 10 µg/ml proteinase K and 10 µg/ml *E. coli* tRNA. After phenol-chloroform extraction and ethanol precipitation, the DNA was suspended in 80% (v/v) formamide containing 0.03% (w/v) bromophenol blue and 0.03% (w/v) xylene cyanol, denatured at 95°C for 3 min and loaded on a standard 6% acrylamide sequencing gel, alongside sequence specific chemical cleavage products of the same fragments.

RESULTS

Identification of two mtDNA fragments which bind to a *P. lividus* mt protein extract

In order to identify mtDNA sequences able to bind to mt proteins, we have developed a strategy for partially purifying proteins which interact with nucleic acids. Embryo mitochondria of *P. lividus* at blastula stage were lysed with Nonidet P40 and loaded on DEAE-Sephacel column. The flow-through was fractionated on a Heparine-Sepharose column eluted with a stepwise ionic strenght gradient. The two peaks of absorbance obtained were

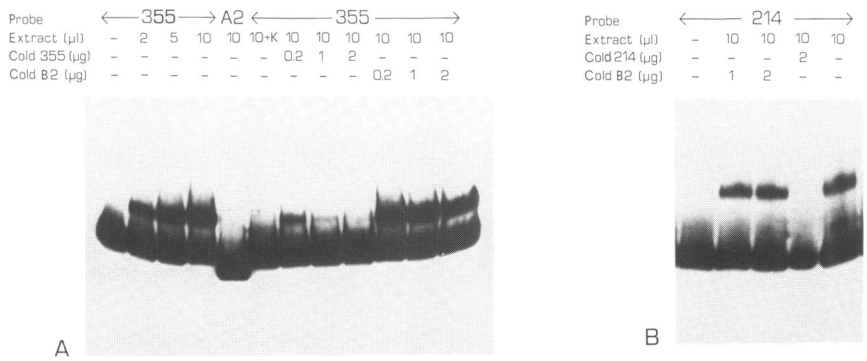

Figure 2. Specificity of the binding to fragments 355 and 214. **A**: Binding to fragment 355. **B**: Binding to fragment 214. About 30,000 cpm (20 ng) of the radioactive fragments were used for each test. A2 is a 330 bp *P. lividus* mtDNA fragment containing the central part of the cytochrome b gene; B2 is a 510 bp fragment containing the central part of the ND5 gene. Proteinase K treatment (10+K lane) was carried out preincubating the extract with 100 µg/ml for 30 min at 37°C.

tested for DNA binding capacity by a gel retardation assay. As shown in Fig. 1A, only the protein fractions eluted at 0.5 M KCl were able to specifically bind to the sea urchin mtDNA. The regions of mtDNA which bind to the protein extract are reported in Fig. 1 (A and B). One is fragment 355, which contains the entire non-coding region and a small part of the tRNA gene cluster; the other is fragment 214 which contains the 3′ region of ND5 and 90 nt downstream the stop codon. Band shift analysis was performed also with other fragments (see Fig. 1B): fragments A, B, C and E were chosen since they contain the consensus sequence TATATATAA, supposed to represent a recognition signal for enzymes involved in transcription or processing (1−3); fragments D and F could contain transcription and replication initiation sites (2−4, 8, 9). None of these fragments was able to bind to the protein extract (results not shown). Fig 2A shows that the binding to 355 is specific: it increases with increasing amounts of the extract; it is not inhibited by the presence of an excess of a heterologous competitor (a fragment of *P. lividus* mtDNA containing the central part of ND5 gene); it is progressively reduced when increasing amounts of cold 355 fragment are added to the reaction mixture. When the extract is treated with proteinase K the retarded band disappears, thus confirming that the electrophoretic delay depends on the formation of a DNA-protein complex. Similar data were obtained with the fragment 214 (Fig. 2B). In order to see whether the binding of the protein extract to the two fragments was somehow related, a cross competition experiment was performed. Fig. 3 shows that when a 200 fold molar excess of cold 355 fragment is added to a binding assay containing the labelled 214 fragment, the retarded band disappears. Similarly a 200 fold molar excess of cold 214 fragment was able to compete with the radioactive 355 fragment in binding to the protein extract. These data suggest that the two fragments bind to the same protein. Filter binding assays using as probe the fragment 355, suggest that one specific mitochondrial protein with a molecular weight of about 40 Kd interacts with the 355 fragment (Roberti et al. in preparation).

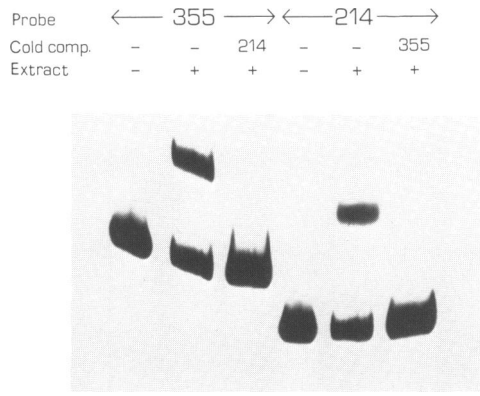

Figure 3. Cross competition between the 355 and 214 fragments. The binding was carried out by adding 16 pmol of cold 214 and 355 fragments to a reaction containing 1 µg of the protein extract and about 50,000 cpm (0.08 pmol) of the labelled fragments.

Binding properties of the fragments 355 and 214

To compare the relative strength of the binding, the dissociation rate of the two preformed protein-DNA complexes was estimated. In this experiment the radioactive 355 and 214 fragments were first incubated with the extract, then a 100 fold molar excess of unlabelled homologous competitor was added. The reactions were stopped at different times and the dissociation rate of the preformed complexes was monitored on band shift gel by determining the rate of disappearance of the protein-bound label. As shown in Fig. 4 (A and B) the dissociation of the protein-214 complex is much faster than that of the protein-355 complex. The results were analyzed according to the integrated pseudo first order equation (13): $\mathbf{ln[CP]/[CPo] = -K_{diss} \times t}$, where **[CP]** represents the concentration of the DNA-protein complex at time **t** and **[CPo]** is the concentration of the complex before the addition of competing DNA. As shown in Fig. 4C the K_{diss} of the complex is 10.8×10^{-5} sec^{-1} for 355 and 6.6×10^{-4} sec^{-1} for 214 corresponding to average lifetimes of about 150 min and 25 min respectively. DNaseI protection experiments were carried out to test whether the DNA binding to the protein is sequence specific. Fig. 5 shows that both fragments bind to the protein in a sequence specific manner. The protected region of fragment 355 comprises positions 1098−1124 on the H-strand and 1100−1126 on the L-strand, corresponding to the first 28 nt of the main non-coding region (Fig. 5A and 5B). Since the protein protects both DNA strands it belongs to the class of double stranded DNA-binding proteins. The fragment 214 shows a protected region of 26 nt (positions 14028−14053 on the H-strand) located 500 nt upstream the 5′ end of the mature cyt b

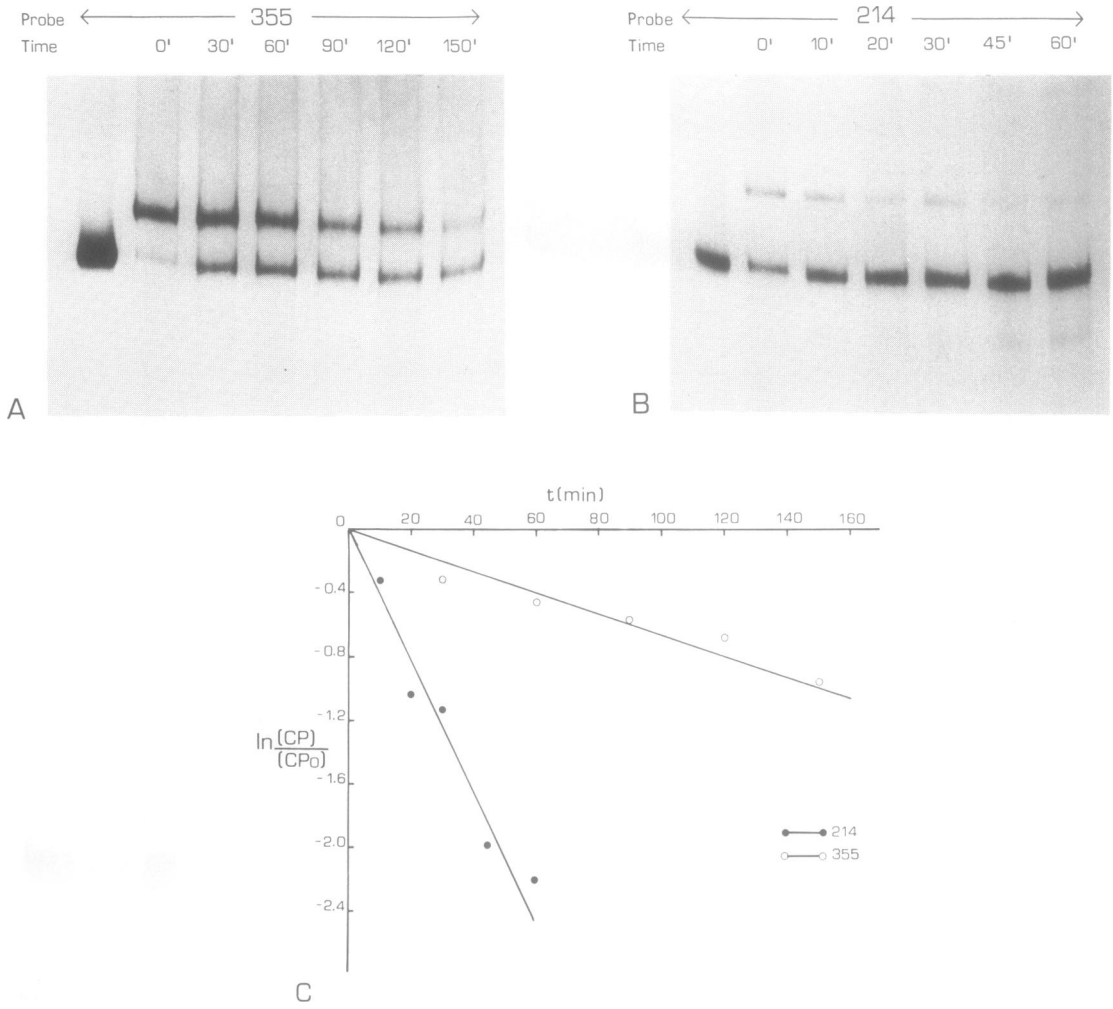

Figure 4. Stability of mt protein-DNA complexes. **A**: Dissociation of the protein-355 complex; **B**: Dissociation of the protein-214 complex. To load all the samples simultaneously on the gel, six binding reactions for each fragment were set up at different times. The 355 and 214 fragments (0.02 pmol) were preincubated with 1 μg of the protein extract for 30 min at 25°C under standard conditions in a total volume of 20 μl. Then 100 fold molar excess of the unlabelled homologous fragment was added to each tube. The reactions were stopped at the indicated times and the samples were loaded on a non-denaturing polyacrylamide gel. The first lane from the left reports the migration of the fragment non-incubated with the protein extract. **C**: Dissociation rate of the DNA-protein complexes formed with 355 and 214 fragments. The intensity of both bound [CP] and unbound [CPo] DNA shown in panels A and B was measured with a LKB XL Laser Densytometer. The values were normalized to the sum of the intensity of bound and unbound DNA at each time point.

mRNA (Fig. 5C). In this case the protection is less evident suggesting that the protein binds more weakly to fragment 214 then to fragment 355. It is likely that the sequence located in fragment 355 is a more efficient target than the one located in fragment 214. The alignment of the protected regions, reported in Fig. 6 shows that they have a high level of similarity: in a tract of 19 bases, 6 of the first 7 nt and 5 of the last 6 nt are identical with an overall homology of about 70%.

DISCUSSION

The data presented in this paper point to the existence of two homologous *P. lividus* mtDNA sequence which bind strongly and specifically to a protein factor having a molecular weight of about 40 Kd. The first sequence is located in the main non-coding region, the second about 500 nt upstream the 5′ end of the cyt b gene. The two binding sequences have in common the motif TTTTRTANNTCYYATCAYA (see Fig. 6). An extensive search on the entire mt genome revealed 51 sequences having a homology with the two binding region comprised between 65% and 78%. However, none of these sequences contains the common motif, thus suggesting that it constitutes an essential requisite for the binding to the protein. It is noteworthy that several of the 51 homologous sequences are located in those fragments that do not bind to the protein extract (Fig. 1B).

It is very likely that the high specificity of the binding reflects a physiological phenomenon affecting mtDNA transcription or replication processes. As the protected regions are situated in the two areas where two transcription initiation sites were located (2, 3, 8, 9), the DNA-binding protein could be a transcription factor. It is well known that all the mtRNA polymerases purified to date, need a factor to catalyze specific mtDNA transcription (14−18). Recent experiments in our laboratory (M. Roberti, unpublished observations) have indicated the presence of a DNA dependent mtRNA polymerase activity in the same Heparine-Sepharose fractions containing the DNA binding protein.

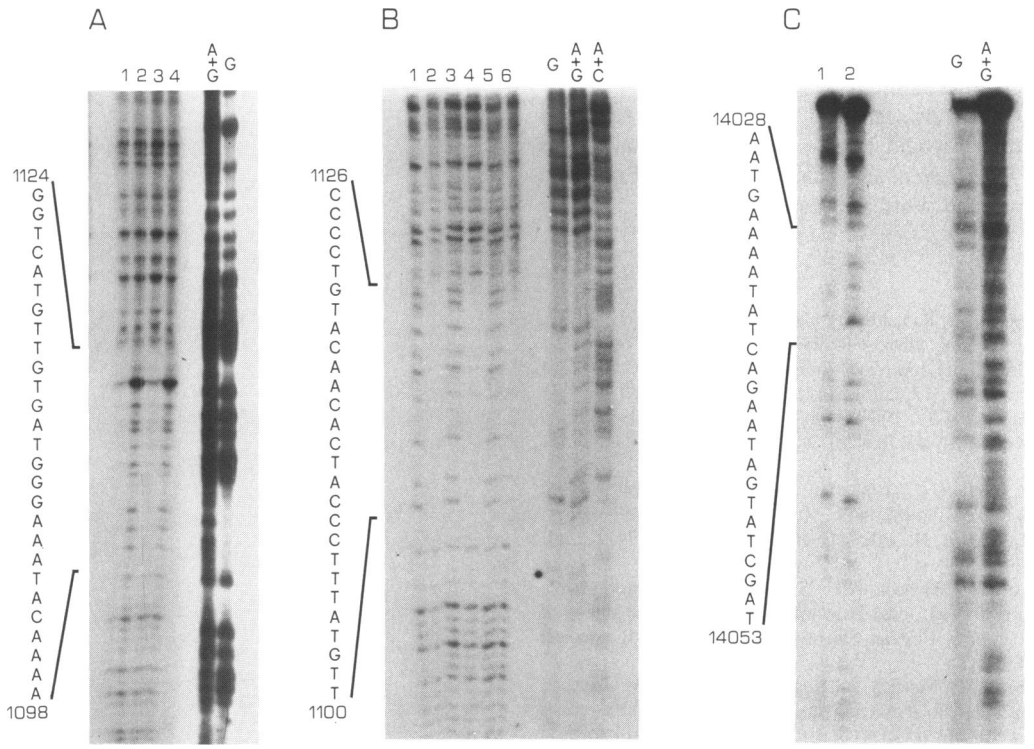

Figure 5. DNaseI protection analysis of the mt protein-DNA complex resolved by gel electrophoresis. The 355 fragment, cloned in the SmaI site of the vector Bluescribe (Stratagene), was 3′ end labelled after digestion of the plasmid with BamHI. Then it was redigested with EcoRI obtaining an end labelled H-strand probe. To prepare the L-strand probe the fragment was labelled at its 5′ end with τ^{32}P ATP and polynucleotide kinase. Then it was digested with AluI, obtaining a fragment of 206 bp (positions 1052−1257). The 214 probe was obtained by labelling the 214 fragment with τ^{32}P ATP and polynucleotide kinase and then digesting with HhaI. The resulting fragment of 156 bp (position 13984−14137) was used as the probe. **A**: DNaseI protection of the complex formed with the H-strand labelled 355 fragment. Lanes 1, 3: DNaseI pattern of DNA-protein complex; lanes 2, 4: DNaseI pattern of uncomplexed free fragment. DNaseI concentration was of 75 μg/ml in lanes 1, 2 and of 100 μg/ml in lanes 3, 4. **B**: DNaseI protection of the complex formed with the L-strand labelled 355 fragment. Lanes 1, 3, 5: DNaseI pattern of uncomplexed free fragment. Lanes 2, 4, 6: DNaseI protection pattern of the complexed DNA. DNAseI concentration was of 50 μg/ml in lanes 1, 2, of 75 μg/ml in lanes 3, 4 and of 100 μg/ml in lanes 5, 6. **C**: DNaseI protection of the complex formed with the H-strand labelled 214 fragment. Lanes 1, 2 show the DNaseI protection pattern of the bound and unbound fragment respectively.

```
1098                                                    1126
   T T T T G T A T T T C C C A T C A C A A C A T G T C C C C
   * * * *   * *     * *     * * * *   *   *
T T A C T T T T A T A G - T C T T A T C A T A G C T A
14028                                                   14053
```

Figure 6. Sequence homology between the 355 (top) and 214 (bottom) protected regions.

However, the mtRNA polymerase activity is not able to specifically transcribe mt templates, suggesting that it may have lost some factor responsible for the specific transcription and that the DNA binding protein identified in this paper is not involved in the mt transcription. This is also supported by the binding properties of the protein. The *P. lividus* factor forms a very stable complex with the target sequences: its half life, even for fragment 214, is of 25 min which is much higher than that reported for the human mt transcription factor (16). Moreover, as shown in the gel retardation experiments reported in Figs. 2 and 3, the two mtDNA fragments are recognized in a very specific manner: there is no affinity for random DNA sequences. This differs from what occurs for other transcription factors (13−18) which tend to sequester even non-specific fragments.

A second possibility is that the DNA-binding protein is involved in mtDNA replication. According to the data reported by Jacobs et al. for the sea urchin *S. purpuratus* (4), the non-coding region of mtDNA contains an origin for H-strand replication and the transition from the RNA primer to DNA occurs at a point which corresponds to the DNaseI protected region of *P. lividus* 355 fragment. Proteins that strictly bind to the origin of mtDNA replication have been described in several systems (19−21). In particular in *Xenopus laevis* mitochondria (19) a double stranded DNA binding protein with sequence specificity for a region containing the replication origin forms relatively stable complexes. Also in HeLa cells a protein attached to the origin of mtDNA replication was identified by Albring et al. (22). In sea urchin the mtDNA replication takes place during the oogenesis, but is blocked in early development (7). The regulation of this phenomenon might involve the selective binding of a protein to the replication origins. In the light of this, it can be hypothesized that during early development the origins would be inaccessible to the replication apparatus, whereas when mtDNA replication is active, the presence of a lower amount of this protein or its chemical modification could prevent the binding to DNA, allowing the mtDNA replication. To test this hypothesis *in vivo* and *in vitro* experimental systems are being developed.

ACKNOWLEDGEMENTS

We are indebted to Prof. C. Saccone for helpful discussion and critical reviewing of the manuscript. This work was partially supported by P.F. 'Ingegneria genetica', CNR (National Research Council). The Authors would like to thank Vito Cataldo and Domenico Munno for excellent technical assistance and Francesca de Palma for typing and word processing.

REFERENCES

1. Cantatore, P., Roberti, M., Rainaldi, G., Gadaleta, M.N. and Saccone, C. (1989) J. Biol. Chem. **264**, 10965–10975.
2. Cantatore, P., Roberti, M., Loguercio Polosa, P., Mustich, A. and Gadaleta, M.N. (1990) Curr. Genet. **17**, 235–245.
3. Elliot, D.J. and Jacobs, H.T. (1989) Mol. Cell. Biol. **9**, 1069–1082.
4. Jacobs, H.T., Herbert, E.R. and Rankine, J. (1989) Nucleic Acids Res. **17**, 8949–8965.
5. Cantatore, P. and Saccone, C. (1987) Int. Rev. Cytol. **108**, 149–208.
6. Attardi, G. and Schatz, G. (1988) Ann. Rev. Cell. Biol. **4**, 289–333.
7. Matsumoto, L., Kasamatsu, H., Piko', L. and Vinograd, J. (1974) J. Cell. Biol. **63**, 146–159.
8. Jacobs, H.T., Elliot, D.J., Mayhook, A.G., Segreto, D.T. and Rinaldi, A.M. (1990) in Quagliariello et al. (eds) Structure, Function and Biogenesis of Energy Transfer Systems. Elsevier Science Publishers B.V. (Biomedical Division) pp. 145–151.
9. Cantatore, P., Roberti, M., Mustich, A., Loguercio Polosa P. and Gadaleta, M.N. (1990) in Quagliariello et al. (eds) Structure, Function and Biogenesis of Energy Transfer Systems. Elsevier Science Publishers B.V. (Biomedical Division) pp. 123–126.
10. Cantatore, P., Nicotra, A., Loria, P. and Saccone, C. (1974) Cell. Diff. **3**, 45–53.
11. Bradford, M.M. (1976) Anal. Biochem. **72**, 248–254.
12. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor University Press, Cold Spring Harbor.
13. Fried, M.G. and Crothers, D.M. (1984) J. Mol. Biol. **172**, 263–282.
14. Fisher, R.P. and Clayton D.A. (1985) J. Biol. Chem. **260**, 11330–11338.
15. Fisher, R.P., Parisi, M.A. and Clayton, D.A. (1989) Genes and Devel. **3**, 2202–2217.
16. Bogenhagen, D.F. and Insdorf, N.F. (1988) Mol. Cell. Biol. **8**, 2910–2916.
17. Winkley, C.S., Keller, M.J. and Jaehning, J.A. (1985) J. Biol. Chem. **260**, 14214–14223.
18. Fisher, R.P. and Clayton, D.A. (1988) Mol. Cell. Biol. **8**, 3496–3509.
19. Cordonnier, A.M., Dunon Bluteau, D. and Brun, G. (1987) Nucleic Acids Res. **15**, 477–490.
20. Mignotte, B. and Barat, M. (1986) Nucleic Acids Res. **14**, 5969–5980.
21. Van Tuyle, G.C. and Pavco, P.A. (1985) J. Cell. Biol. **100**, 251–257.
22. Albring, M., Griffith, J. and Attardi, G. (1977) Proc. Natl. Acad. Sci. USA **74**, 1348–1352.