The 25 kDa protein recognizing the rat curved region upstream of the origin of the L-strand replication is the rat homologue of the human mitochondrial transcription factor A

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Abstract Mass spectrometry matrix-assisted laser desorption ionization (MALDI) analysis and N-terminus sequencing as well as immunoblotting experiments using human and mouse antibodies have allowed us to identify the 25 kDa protein, previously isolated from rat liver using magnetic beads coated with a rat liver mitochondrial (mt) DNA region upstream of the Ori-L, as the homologue of human mt transcription factor A (mtTFA). We can therefore identify this DNA binding protein as the rat mtTFA. Furthermore, since we previously showed that the 25 kDa protein purified from rat liver was able to bind the curved mtDNA region upstream of the Ori-L as well as the curved mtDNA in the D-loop region, the results here reported lead us to state, for the first time, that mtTFA binds both the curved regions of mtDNA upstream of the two replication origins.

Key words: Mitochondrial DNA binding protein;
N-Terminus sequencing;
Rat mitochondrial transcription factor A

1. Introduction

In mammals, the mitochondrial (mt) genome exists as a closed circular, double-stranded DNA molecule of about 16 kb in length whose compact gene organization is highly conserved [1–5]. The majority of regulatory sequences responsible for controlling expression of mammalian mtDNA are located in the D-loop containing region. This region contains the origin of heavy strand replication (Ori-H) and the promoters for the transcription of the two strands (HSP and LSP). Another very small non-coding tract consisting of only 30 nucleotides is located 10 kb clockwise from the first and contains the replication origin of the light strand (Ori-L). Communication between the nuclear and mt genetic systems in response to cellular metabolism and energetic requirements regulates the mt biogenesis [6]. Mammalian mt promoters have a bipartite structure consisting of a small initiation site [7,8] and a recognition domain for mt transcription factor A (mtTFA) located approximately −10 to −40 bp upstream from the RNA start site [9,10]. mtTFA is a key activator of mt transcription in mammals but is also implicated in mtDNA replication, since transcription from the major mt promoter generates a RNA primer necessary for initiation of mtDNA replication [11–12]. In mammals, this factor has been isolated from human (h-mtTFA) [13] and mouse (m-mtTFA) [14]. In mouse, Larsson and co-workers [14] have reported that a single gene encodes two isoforms, one of which is imported into the nucleus rather than into mitochondria. The nuclear m-mtTFA isoform, known as 'tsHMG protein' is absent from somatic cells, whereas it is the predominant form in male germ cells functioning as a testis-specific transcription activator or participating in nuclear chromatin condensation. The involvement of m-mtTFA (also called Tfam by the authors) in regulation of the mtDNA copy number and its requirement for mt biogenesis and embryonic development have been demonstrated in knockout mice [15]. h-mtTFA has been shown to contain two high-mobility group (HMG)-like domains, similar to those found in yeast mtDNA binding factor ABF2 (also termed sc-mtTFA), presumably involved through DNA packaging in mt replication and transcription [7,16,17]. In vitro DNA binding studies have demonstrated that h-mtTFA is flexible in its recognition of DNA sequences since, at saturating concentrations, it binds multiple sites within the D-loop region. These observations, together with the finding that h-mtTFA can bend and wrap the D-loop region of mtDNA [13,18], have led to the proposal that the binding of mtTFA to the mtDNA may be of use to organize or package the major regulatory region of mtDNA in a way necessary for mtDNA replication and transcription [19]. No information has been reported until now about the binding of mtTFA to regions of mtDNA outside of the D-loop region. Ghivizzani et al. [20] used in organello footprinting experiments in bovine to assay the Ori-L region of mtDNA and the surrounding tRNA genes for protein binding, but they were unable to detect any significant alteration indicative of protein binding in these mtDNA regions.

As far as rat is concerned, DNA-protein interactions to multiple sites within the D-loop region, presumably due to the analogue of h-mtTFA, as well as the existence of a DNA-protein interaction at the ND2 region of rat mtDNA have been demonstrated by in organello footprinting [21–22]. We previously purified a 25 kDa mtDNA binding protein from rat liver using magnetic beads coated with a rat mtDNA curved region within the ND2 gene upstream of Ori-l [23]. Furthermore, we showed that this protein was also able to bind the rat curved mtDNA region close to Ori-H in vitro and the curved h-mtDNA within the ND2 gene upstream of Ori-L [23]. In order to identify this rat 25 kDa mtDNA binding protein, we performed matrix-assisted laser desorption ionization (MALDI) analysis of its tryptic peptides, sequencing of the N-terminus of the protein and immunoblotting experiments with mouse and human antibodies for mtTFA. In this paper, we report the results of these analyses which allow us to identify this protein as the rat homologue of h-mtTFA. Furthermore, since we have previously demonstrated that this rat 25 kDa protein binds the curved mtDNA region

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upstream of Ori-L [23], the data here reported are the first demonstration that mtTFA binds the curved region of mtDNA upstream of Ori-L as well as the curved region in the D-loop [23].

2. Materials and methods

2.1. Purification of the rat mt 25 kDa protein

The isolation and purification of this protein was carried out by Sepharose CL-6B chromatography followed by magnetic DNA affinity chromatography as described in [23].

2.2. Sequencing and MALDI analysis

Sequencing of the N-terminus of the rat 25 kDa mtDNA binding protein and MALDI analysis were carried out by the Mass Spec service of the Protein and Peptide group in EMBL (Heidelberg, Germany). Several runs of MALDI analysis were performed. The tryptic fragments characterized using MALDI in each run were compared with the 'NRDB' database that is available at http://www.embl-heidelberg.de/srs5bin.

2.3. Sodium dodecyl sulfate-polyacrylammide gel electrophoresis (SDS-PAGE) and Western blotting

Gel electrophoresis was carried out as described by Laemmli [24] on a 13% SDS-PAGE slab gel. The transfer procedure on a Immobilon P-membrane has been adapted from Burnette [25] using a MULTIPHOR II NovaBlot (2177-250). The membrane was briefly stained for 2 min with Coomassie brilliant blue R-250 as described [26,27] to check the efficiency of the transfer. Immediately following transfer, nitrocellulose sheets were immersed in blotting buffer containing 5% non-fat dry milk, 250 mM NaCl, 1% Triton X-100 and 20 mM Tris-HCl, pH 7.5, for 60 min on a rocking platform. Then, the reaction with the appropriate immune serum diluted from 1:1000 to 1:3000 in blotting buffer was carried out for 2 h or overnight at 4°C. Membranes were then incubated for 60 min with alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma) diluted 1:5000 in blotting buffer and revealed for alkaline phosphatase using 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.48 mM nitro blue tetrazolium in 10 mM Tris-HCl, pH 9.5 (Jansen Pharmaceutica).

2.4. Polyclonal antibodies

Anti-h-mtTFA antibodies were kindly provided by Dr Rudolf J. Wiesner (Köln, Germany) and anti-rat mtTFA antibodies were kindly provided by Dr Nils-Göran Larsson (Stockholm, Sweden).

3. Results and discussion

The regulation of mtDNA expression is crucial for mt biogenesis. Specific DNA binding proteins are known to be important regulators of mtDNA transcription and replication. In particular, mtTFA is the first identified mammalian transcription factor of mtDNA [10]. Furthermore, it regulates the mtDNA copy number in vivo and it is essential for mt biogenesis and embryonic development [15]. mtTFA has been

Name: HMGT_MOUSE TESTIS –SPECIFIC HIGH
Accession: swiss | P40630
Number of amino acids: 199
Protein mass (average): 23384.04 Da
PI: 10.54

MGSYPKKPMSSYLRFSTEQLPKFKAKHPDAKLSELVRKIAALWRELPEAEKKVYEADFKAEWKAYKEAVSKYKEQLTPSQLMGMEKEAR
QRRLKKKALVKRRELILLGKPKRPRSAYNIYVSESFQEAKDDSAQGKLKLVNEAWKNLSPEEKQAYIQLAKDDRIRYDNEMKSWEEQMAE
VGRSDLIRRSVKRSGDISEH

Search Parameters:
Cut after K, R but not before P
Mass Accuracy: 0.0100 %
Mass is monoisotopic mass

Cys is CYS_CARBAMIDOMETHYL. Met is NORMAL.

Measured	Calculated	(+ H)	Diff.	Residues	Sequence
934.553	934.536	(mono)	0.017	153-160	(K)QAYIQLAK(D)
949.495	949.499	(mono)	-0.004	15-22	(R)FSTEQLPK (F)
981.511	981.519	(mono)	-0.007	7-14	(K)KPMSSYLR(F)
1010.663	1010.661	(mono)	0.002	103-111	(R)ELILLGKPK(R)
1100.637	1100.646	(mono)	-0.009	137-145	(K)LKLVNEAWK(N)
1186.650	1186.647	(mono)	0.004	64-73	(K)AYKEAVSKYK(E)
1320.661	1320.691	(mono)	-0.029	153-163	(K)QAQYIQLAKDDR(I)
1321.646	1321.584	(mono)	0.062	172-182	(K)SWEEQMAEVGR(S)
1385.649	1385.674	(mono)	-0.025	53-63	(K)VYEADFKAEWK(A)
1735.765	1735.817	(mono)	-0.052	115-129	(R)SAYNIYVSESFQEAK(D)
2145.093	2145.072	(mono)	0.021	112-129	(K)RPRSAYNIYVSESFQEAK (D)

These 11 peptides cover 95 out of 199 Amino Acids (47.74 %)

Cys is CYS_ CARBAMIDOMETHYL. Met is METHIONINE_ OXIDIZED.

Measured	Calculated	(+ H)	Diff.	Residues	Sequence
997.510	997.514	(mono)	-0.003	7-14	(K)KPMSSYLR(F)
1084.492	1084.509	(mono)	-0.018	164-171	(R)IRYDNEMK(S)
1337.562	1337.579	(mono)	-0.017	172-182	(K)SWEEQMAEVGR(S)

These 3 peptides cover 27 out of 199 Amino Acids (13.57%)

No peptides matched for: Cys is CYS_ ACRYLAMIDE. Met is NORMAL.
No peptides matched for: Cys is CYS_ ACRYLAMIDE. Met is METHIONINE_ OXIDIZED.
Statistics for all:
These 14 peptides cover 103 out of 199 Amino acids (51.76 %).

Fig. 1. Comparison of the tryptic fragments characterized by MALDI versus the 'NRDB' database. The figure shows a representative run of MALDI analysis of tryptic fragments of the rat 25 kDa mtDNA binding protein compared with the tryptic fragments of tsHMG.

isolated from mouse and human as a 25 kDa protein interacting with the D-loop control region [13,14].

previously, we reported the isolation from rat liver mitochondria of a 25 kDa mtDNA binding protein with the capability to bind the curved regions present in the rat mt genome upstream of Ori-L and Ori-H [23].

To identify this nuclear-encoded mt protein, we carried out several runs of tryptic digestions of the rat 25 kDa mtDNA binding protein that were analyzed using the mass spectrometry MALDI technique and compared, on the basis of their molecular weight, with the 'NRDB' database. The comparison of the tryptic fragments characterized by MALDI with the 'NRDB' database indicated that the identified tryptic fragments of this 25 kDa rat mtDNA binding protein identified in each run were always identical with those of nuclear and mature forms of m-mtTFA and of the tsHMG protein isolated from mouse spermatids. In fact, the two mouse proteins, mtTFA and tsHMG, are encoded by the same gene. The m-mtTFA gene consists of seven exons (I–VII) plus a short alternative first exon [14]. In Fig. 1, we report the results of a representative MALDI analysis in which the tryptic fragments of the rat protein have been compared with the tsHMG fragments. The underlined fragments are those identified in the run reported in Fig. 1 as the same in our protein and in tsHMG.

Thereafter, we carried out sequencing of the N-terminus of the rat liver protein eluted using magnetic beads and compared the sequence of the first 17 amino acids identified with the complete protein database using the BLAST program [28]. We aligned the 17 N-terminus residues of the rat 25 kDa protein with the sequence of tsHMG and h- and m-mtTFA reported in the literature. The analysis revealed (Fig. 2) a significant similarity between the N-terminal 17 amino acid residues identified with the second exon of tsHMG protein and the mature form of m- and h-mtTFA, showing a high degree of conservation. These results reinforced the results of the MALDI analysis, suggesting that the mtDNA 25 kDa binding protein might be the rat homologue of h- and m-mtTFA.

To identify the 25 kDa rat mtDNA binding protein as the rat mtTFA, we assayed the 25 kDa protein by Western blot, before and after magnetic bioseparation, with polyclonal anti-mtTFA antibodies from two different species. The Coomassie blue-stained Western blots of molecular weight markers, the protein purified by magnetic bioseparation and the heparin fraction containing the 25 kDa protein are reported in Fig. 3 (lanes 1, 2 and 3, respectively). The corresponding immunoblots at a 1:3000 dilution of mouse and human antibodies are reported, respectively, in the subsequent slots. Western blot analysis revealed that antibodies against m- and h-mtTFA detected a single protein band of 25 kDa both before and after the purification of the heparin extract.

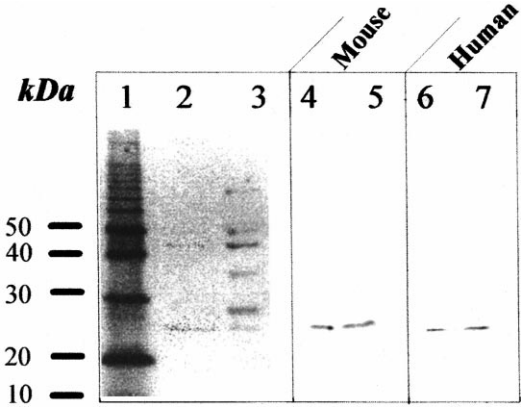

Fig. 2. Sequences alignment of the rat 25 kDa mtDNA binding protein with h-mtTFA (X64269) and m-mtTFA (U57939) and testis-specific HMG protein (U63858). The results of the analysis of the sequence of the 17 N-terminal amino acid residues of the rat 25 kDa mtDNA binding protein are reported. The sequence comparison has been performed starting at exon II from which the compared proteins are identical. The gen EMBL accession numbers of the compared sequences are reported in brackets.

Fig. 3. Coomassie brilliant blue and immunoblotting of 25 kDa rat mtDNA binding protein with anti-m- and anti-h-mtTFA antibodies. Lane 1: molecular weight markers from bottom to top are 10, 20, 30, 40 and 50 kDa proteins. Lane 2: rat 25 kDa mtDNA binding protein after magnetic bioseparation (0.4 μg protein). Lane 3: heparin fraction (4 μg proteins) containing rat mt 25 kDa mtDNA binding protein. Lane 4 and 5: immunoblots of 25 kDa rat mtDNA binding protein after and before, respectively, magnetic bioseparation probed with anti-m-mtTFA. Lanes 6 and 7: immunoblots of 25 kDa rat mtDNA binding protein after and before, respectively, magnetic bioseparation probed with anti-h-mtTFA.

In conclusion, on the basis of the data here reported, we can say that the 25 kDa mtDNA binding protein, isolated by using the curved region in the ND2 gene upstream of the regulatory region containing the Ori-L of rat mtDNA as a ligand, is mtTFA and we have named it r-mtTFA.

Furthermore, since we previously reported that the 25 kDa protein, now identified as r-mtTFA, was able to bind a curved region upstream of Ori-L as well as the curved mtDNA in the D-loop containing region [26], the data reported here allow us to state, for the first time, that r-mtTFA interacts with both the curved regions of mtDNA upstream of the two replication origins. Whether the binding of r-mtTFA to the curved mtDNA region near Ori-L has a regulatory significance or is just one of the several sequences bound by r-mtTFA deserves further analysis.

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References

[1] Anderson, S., Banker, A.T., Barrel, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F. and Schreier, P.H. (1981) Nature 290, 457–465.

[2] Bibb, M.J., Van Etten, R.A., Wright, C.T., Warberg, M.W. and Clayton, D.A. (1981) Cell 26, 167–180.

[3] Anderson, S., de Bruijn, M.H.L., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, I.G. (1982) J. Mol. Biol. 156, 683–717.
[4] Gadaleta, G., Pepe, G., de Candia, G., Quagliariello, C., Sbisa', E. and Saccone, C. (1989) J. Mol. Evol. 28, 497–516.
[5] Arnason, U., Gullberg, A. and Widegren, B. (1991) J. Mol. Evol. 33, 556–568.
[6] Saccone, C. and Sbisà, E. (1994) in: Principles of Medical Biology, Vol. 1B, pp. 37–70, Edit E.E. Bitter Jai Press, Greenwich, CT.
[7] Chang, D.D. and Clayton, D.A. (1984) Cell 36, 635–643.
[8] Chang, D.D. and Clayton, D.A. (1986) Mol. Cell Biol. 6, 3252–3261.
[9] Fisher, R.P., Parisi, M.A. and Clayton, D.A. (1989) Genes Dev. 3, 2202–2217.
[10] Fisher, R.P., Topper, J.N. and Clayton, D.A. (1987) Cell 50, 247–258.
[11] Parisi, M.A. and Clayton, D.A. (1991) Science 252, 965–969.
[12] Dairaghi, D.J., Shadel, G.S. and Clayton, D.A. (1995) J. Mol. Biol. 249, 11–28.
[13] Fisher, R.P. and Clayton, D.A. (1988) Mol. Cell Biol. 8, 3496–3500.
[14] Larsson, N., Garman, J.D., Oldfors, A., Barsh, G. and Clayton, D.A. (1996) Nat. Genet. 13, 296–302.
[15] Larsson, N., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. and Clayton, D. (1998) Nat. Genet. 18, 231–236.
[16] Diffley, J.F.X. and Stillman, B. (1991) Proc. Natl. Acad. Sci. USA 88, 7864–7868.
[17] Duffels, J.F.X. and Stillman, B. (1992) J. Biol. Chem. 267, 3368–3374.
[18] Fisher, R.P., Lisowsky, T., Parisi, M.A. and Clayton, D.A. (1992) J. Biol. Chem. 267, 3358–3367.
[19] Ghivizzani, S.C., Madsen, C.S., Nelen, M.R., Ammini, C.V. and Hauswirth, W.W. (1994) Mol. Cell Biol. 14, 7717–7730.
[20] Ghivizzani, S.C., Madsen, C.S. and Hauswirth, W.W. (1993) J. Biol. Chem. 268, 8675–8682.
[21] Cantatore, P., Daddabbo, L., Fracasso, F. and Gadaleta, M.N. (1995) J. Biol. Chem. 270, 25020–25027.
[22] Cingolani, G., Capaccio, L., D'Elia, D. and Gadaleta, G. (1997) Biochem. Biophys. Res. Commun. 231, 856–860.
[23] Gadaleta, G., D'Elia, D., Capaccio, L., Saccone, C. and Pepe, G. (1996) J. Biol. Chem. 271, 13537–13541.
[24] Laemmli, O.K. (1970) Nature 227, 680–685.
[25] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[26] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[27] Scaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–509.
[28] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.