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Protein Binding to a Single Termination-Associated Sequence in the Mitochondrial DNA D-Loop Region

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A methylation protection assay was used in a novel manner to demonstrate a specific bovine protein-mitochondrial DNA (mtDNA) interaction within the organelle (in organello). The protected domain, located near the D-loop 3′ end, encompasses a conserved termination-associated sequence (TAS) element which is thought to be involved in the regulation of mtDNA synthesis. In vitro footprinting studies using a bovine mitochondrial extract and a series of deleted mtDNA templates identified a ~48-kDa protein which binds specifically to a single TAS element also protected within the mitochondrion. Because other TAS-like elements located in close proximity to the protected region did not footprint, protein binding appears to be highly sequence specific. The in organello and in vitro data, together, provide evidence that D-loop formation is likely to be mediated, at least in part, through a *trans*-acting factor binding to a conserved sequence element located 58 bp upstream of the D-loop 3′ end.

The mammalian mitochondrial genome is a closed circular, double-stranded DNA of approximately 16 kb. The number of mitochondrial DNA (mtDNA) molecules per cell varies depending upon cell type and respiratory capacity, indicating that mtDNA synthesis is a highly regulated process. mtDNA copy number can range from approximately 700 in mouse L-cell fibroblasts (41) to about 400 times higher in mammalian oocytes (36). Highly oxidative tissues such as heart muscle contain a significantly greater number of mitochondrial genomes than do the more glycolytic type II skeletal muscles (3). The oxidative capacity of a given cell is not static; even limiting amounts of exercise result in an increase in both mitochondrial mass and volume relative to other cellular constituents (29, 31). Additionally, it has been demonstrated that chronic stimulation of normally weakly oxidative skeletal muscle results in a concomitant increase in cellular mtDNA content (47, 48). mtDNA content in logarithmically growing mouse tissue culture cells increases proportionally to total cellular DNA, further evidence that mtDNA synthesis is a regulated process (45). Although little is known about cellular control of mitochondrial gene copy number, it has been suggested that key regulatory sites are likely to be associated with mtDNA replication and reside within the mtDNA molecule itself (12, 15).

The process of mtDNA replication has been recently reviewed (12, 13). Briefly, mtDNA replication appears to involve only leading-strand DNA synthesis and begins at the unidirectional origin of heavy (H)-strand synthesis (O_H), utilizing an RNA primer transcribed from the light (L)-strand promoter. H-strand DNA elongation continues around the entire length of the genome, with L-strand DNA synthesis beginning at a separate unidirectional origin (O_L) only after displacement of the template strand. Interestingly, only a few initiation events at O_H actually result in the synthesis of a full-length genome; nearly 95% are terminated at specific sites, depending on the vertebrate species, 500 to 1,000 bases downstream (5). This finding suggests that the site where H-strand DNA synthesis is either prematurely terminated or allowed to continue functions as a regulatory domain for mtDNA replication. Because the newly synthesized, terminated strand remains annealed to the supercoiled template, a triplex DNA structure known as the displacement (D) loop is created. Whether the D-loop strand 3′-hydroxyl end functions as a reinitiation site for continued synthesis is unknown (12), but a high level of D-loop strand turnover suggests that most do not. The identification of a conserved 15-bp sequence located a short distance upstream from the 3′ D-loop ends in human and mouse cells led to the proposal that arrest of H-strand synthesis is a template-directed event (14). Consistent with this suggestion, closely related termination-associated sequences (TASs) have been identified at similar positions relative to mapped D-loop DNA 3′ ends in *Xenopus laevis* (15), cow, pig (34), and several primates (21).

The current picture, therefore, is that conserved sequence domains (TAS elements) located short distances upstream of 3′ ends of D-loop DNA exist in all vertebrate species and are candidate *cis* elements for the regulation of H-strand DNA synthesis. However, no experimental evidence regarding the potential function of TAS elements exists. Additionally, it remains unclear how such elements might function as terminators of H-strand DNA synthesis both because the number of consensus TAS domains can exceed the number of 3′ D-loop termini and because TASs are found at variable distances (20 to 80 bp) upstream of these termination sites.

In this study, we have adapted the methylation protection technique to assay purified bovine mitochondria for specific protein-mtDNA interactions near the D-loop 3′ end in an in vivo-like environment. By this method, we identified a single protected sequence element located 58 bases upstream from the D-loop 3′ end. Using partially purified protein extracts from bovine mitochondria, a 48-kDa protein(s) which binds specifically to this element was identified. In vitro DNase I footprint experiments using a series of deleted target sequences demonstrated that although seven TAS-like sequences exist in the region, only one is selectively bound. These results suggest that D-loop termination and, subsequently, mtDNA copy number may be regulated through the binding of a sequence-specific protein factor.

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MATERIALS AND METHODS

Isolation of mitochondria and protein purification. Mitochondria were isolated from bovine brain tissue by differential centrifugation as previously described (25). Mitochondrial extract was prepared essentially as described by Walberg and Clayton (46); 1 g of purified mitochondria (wet pellet) was resuspended in 6.0 ml of 2× buffer A (buffer A is 20 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 7.5% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM benzamide) at 4°C. The volume was adjusted to 8.4 ml with 4°C water. After addition of 1.2 ml of 20% Triton X-100, the mitochondria were homogenized by 10 strokes in a glass homogenizer. The homogenization was repeated upon addition of 3.6 ml of 4 M KCl, and the lysate was cleared by centrifugation in a Beckman Ti-50 rotor for 30 min at 40,000 rpm at 4°C. The lysate was diluted fourfold with buffer A to adjust the KCl concentration to 0.2 M and loaded onto a 40-ml heparin-Sepharose column (Pharmacia, Inc.). After the column was washed with 3 column volumes of buffer A plus 0.2 M KCl, bound proteins were eluted in buffer A plus 0.6 M KCl. Fractions containing protein were pooled and dialyzed against 1 liter of buffer A for 2 h at 4°C. Once the concentration of KCl in the sample was below 50 mM, as determined by conductivity, the sample was loaded onto a 25-ml DEAE-Sephacel column (Pharmacia) which had been previously equilibrated in buffer A plus 50 mM KCl. After the column was washed with 3 column volumes of buffer A plus 50 mM KCl, proteins were eluted with a 30-ml linear gradient of KCl (from 0.05 to 0.5 M) and collected in 2-ml fractions. Following determination of KCl concentrations, fractions were dialyzed for 12 h at 4°C against 10 mM Tris-HCl (pH 8.0)–5 mM $MgCl_2$–0.1 mM EDTA–10 mM KCl–10% glycerol. Protein concentrations were measured by the method of Bradford (6). DNA binding activity of each fraction was determined by using the DNase I protection assay (see below). Peak binding activity eluted from the DEAE-Sephacel column at ~150 mM KCl.

DNase I protection. All binding reactions and DNase I digestions were performed essentially as described by Galas and Schmitz (22). In a 15-μl reaction, various amounts of DEAE-Sephacel protein fraction were incubated with a ^{32}P-end-labeled DNA fragment (~10 ng) at room temperature for 10 min in binding buffer (12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 48 mM NaCl, 5 mM $MgCl_2$, 4 mM Tris-HCl [pH 7.5], 0.6 mM EDTA, 0.6 mM dithiothreitol) and subjected to partial DNase I cleavage by the addition of 15 μl of 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.2 to 0.5 μg of DNase I per ml. Following the addition of 150 μl of DNase I stop buffer (0.33 M sodium acetate [pH 5.2], 11 mM EDTA, 1% sodium dodecyl sulfate [SDS], 60 μg of tRNA per ml), samples were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and subjected to electrophoresis on either 6 or 8% polyacrylamide–7.5 M urea sequencing gels. Many DNA probes and footprinting targets used in this assay were prepared from clone pBS314, which contains the bovine mtDNA *Bam*HI-*Ssp*I fragment described in the text, or from subclones of pBS314 which contain selected portions of this sequence. In two cases, oligonucleotides were synthesized (sequences are described in the text), cloned into the Bluescript(+) phagemid vector (Stratagene), and used for probe preparation. Standard techniques were used for plasmid DNA preparation, labeling, cloning (43), and sequencing (44).

In organello footprinting. The technique described here for footprinting of mtDNA within mammalian mitochondria (in organello) was adapted from previously described methods used to analyze nuclear genes in vivo (4, 11, 17, 30). Mitochondrial pellets (isolated from bovine brain tissue as described above) with wet weights of 0.2 g were resuspended in 0.2 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $NaPO_4$, 1.4 mM KPO_4 [pH 7.4]) at 37°C. Dimethylsulfate (DMS) was added to a concentration of 0.5%, and each sample was incubated for various times (2.5 to 5.0 min) at room temperature. Immediately following incubation, the samples were placed on ice, and 1 ml of 4°C PBS was added. The mitochondria were repelleted by centrifugation for 1 min at 10,000 × *g*. After two more washes in 4°C PBS, the pellets were resuspended in 400 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 25 mM EDTA, 200 mM NaCl) and 25 μl of 10% SDS was added. The samples were incubated at room temperature for 5 min, extracted twice with equal volumes of phenol, twice with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform, and ethanol precipitated. Control samples of mtDNA were also prepared as described above, omitting the DMS treatment. To create a defined DNA end from which to map footprinted regions, both control and test mtDNA samples were digested with *Bam*HI in a volume of 100 μl according to the specifications of the manufacturer (Promega). After digestion, an equal volume of 100 mM sodium cacodylate (pH 7.0) was added to the control sample, after which DMS (final concentration, 0.5%) was added. The samples were incubated for 3 min at room temperature, and the reaction was terminated by addition of 50 μl of 4°C DMS stop buffer (1.5 M sodium acetate [pH 7.4], 1 M β-mercaptoethanol). Both control and test samples were ethanol precipitated and subjected to piperidine cleavage, using standard methods (35). mtDNA samples (1 μg) were either resolved on 6% polyacrylamide–7.5 M urea sequencing gels and electroblotted to Hybond N+ (Amersham) for Southern blot analysis or analyzed by primer extension.

Southern hybridization and primer extension analysis. For Southern blotting, RNA probes complementary to the region and strand of interest were generated by using either the T7 or T3 RNA polymerase and the corresponding promoter in the clone pBS314 according to the specifications of the manufacturer (United States Biochemical). Hybridization protocols were essentially as described previously (11) except that incubations were carried out overnight at 65°C in a rolling-bottle hybridization oven (Robbins Scientific). For primer extension analysis, 1 μg of either control or test mtDNA fragments was extended by using an end-labeled primer (1 pmol) complementary to L-strand nucleotides 16186 to 16205 (WH202; 5'-GATCCCTGCCTAGCGGGT TG-3') in a unidirectional reaction using *Taq* polymerase (Perkin-Elmer/Cetus). Following 10 cycles (1 min at 94°C, 2 min at 55°C, and 5 min at 72°C), the samples were extracted with phenol-chloroform, ethanol precipitated, and analyzed by electrophoresis on a 6% polyacrylamide–7.5 M urea sequencing gel.

In vitro methylation protection assay. Protein-DNA binding reactions were performed as described for the DNase I assay except that 50 mM sodium cacodylate (pH 7.0) was used instead of Tris-HCl in the binding buffer. Samples were incubated at room temperature in the presence of DMS (0.5%) for 2 to 5 min, and the reaction was quenched by the addition of DMS stop buffer plus tRNA (60 μg/ml). Piperidine cleavage and nucleic acid preparation were performed as described above.

UV cross-linking. Uniformly labeled probe DNA was

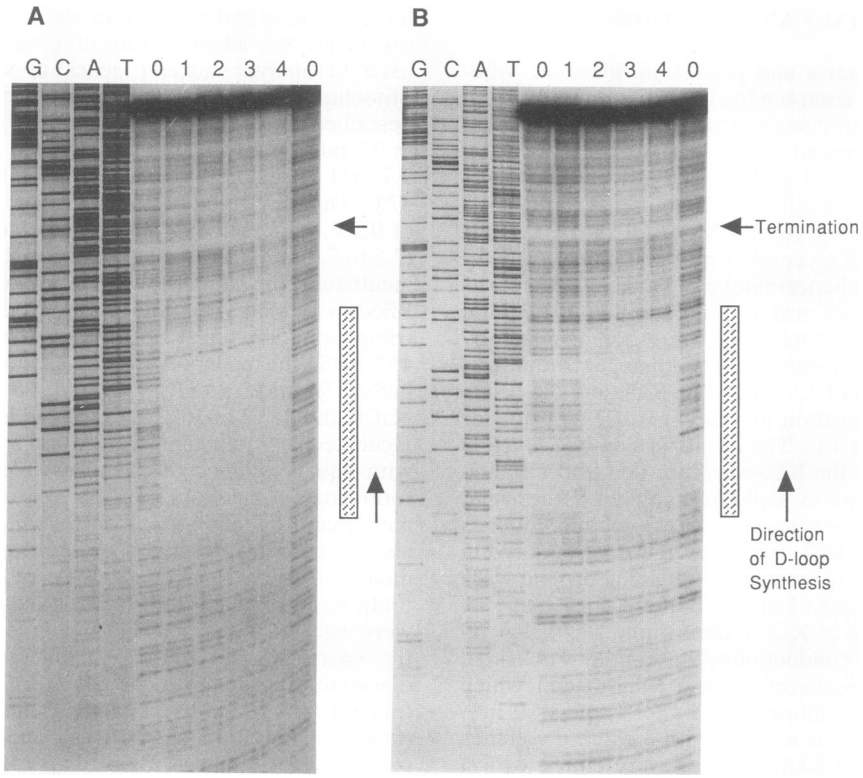

FIG. 1. DNase I analysis of mitochondrial protein binding near the mtDNA D-loop 3′-terminal region. The mtDNA 314-bp *Ssp*I-*Bam*HI fragment, which spans the D-loop 3′-end region, was end labeled on either the L strand (A) or H strand (B) and incubated with 0 to 4 μl of DEAE-Sephacel-purified mitochondrial protein fraction (10 μg/ml) as indicated above each lane. After digestion with DNase I, products were analyzed on 6% polyacrylamide denaturing gels alongside corresponding dideoxy sequencing reactions complementary to the L strand (lanes G, C, A, and T). A protected region (~67 bp) located approximately 35 bp upstream of the D-loop termination site is indicated by a stippled bar for both strands. The direction of H-strand synthesis and the D-loop 3′ end are also shown.

prepared by amplification of either clone pKS20 (described in the text) or pBluescript vector sequences only, using *Taq* polymerase and primers complementary to the T3 and T7 RNA promoter sequences in a standard polymerase chain reaction (PCR). The PCR mixture contained [α-^{32}P]dCTP and [α-^{32}P]dGTP (0.5 μM each) along with unlabeled nucleotides (dCTP and dGTP [2 μM each] and dATP and dTTP [200 μM each]). Probes were purified on a 4% nondenaturing polyacrylamide gel and eluted from the gel overnight in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Probe specific activity was determined by liquid scintillation, and the amount of incorporated versus free label was calculated on a PhosphorImager (Molecular Dynamics, Inc.). Unlabeled competitor DNAs were generated as described above without radiolabeled nucleotides. DNA concentrations were determined spectrophotometrically.

Cross-linking experiments were performed as described by Chodosh et al. (9) except that DNase I binding buffer was used and irradiation was performed on ice at a distance of 10 cm from the UV source in a UV-Stratalinker (Stratagene). Following irradiation at 1.2×10^5 μJ/cm^2/min, protein (80 ng)-DNA (1 ng) samples were adjusted to 10 mM $CaCl_2$ and digested for 30 min at 37°C with 3.3 U of DNase I (Bethesda Research Laboratories) and 1 U of micrococcal nuclease (Sigma). Samples and molecular weight markers were resolved on an SDS–10% polyacrylamide gel (33) and electroblotted onto a nitrocellulose membrane (0.2-μm pore size). Protein molecular weight markers were visualized by staining with amido black, and radioactivity was quantitated on a PhosphorImager.

RESULTS

In vitro footprinting of the bovine mitochondrial 3′ D-loop region. The 3′ end of the D loop has been mapped to a triplet of adjacent nucleotides centered at position 16023 (23) (numbering is according to Anderson et al. [1]). Nearby, multiple sequence elements conforming to a TAS consensus have been noted previously (34) (see Fig. 2). To test for specific protein binding near the D-loop 3′ end, the insert from clone pBS314 (the 314-bp *Ssp*I-*Bam*HI bovine mtDNA fragment, nucleotides 15892 to 16205, encompassing the D-loop termination site and seven TAS-like elements) was incubated with a chromatographic fraction containing mitochondrial protein and assayed for DNase I protection. Figure 1 reveals the protection of a specific mtDNA sequence, approximately 67 bp in length on both L and H DNA strands, that begins near nucleotide 16060 and extends to 16127. The proximal end of the footprinted region resides 35 bp upstream of the D-loop termination site and encompasses three TAS-like sequences that we have labeled TAS-A, -B, and -C (Fig. 2). Outside the protected region, there are four other TAS-like sequences (TAS-D through TAS-G) which did not footprint, suggesting selectivity of binding for a subset of TAS domains.

In organello footprinting of the bovine mitochondrial 3′ D-loop region. To determine whether the in vitro footprint

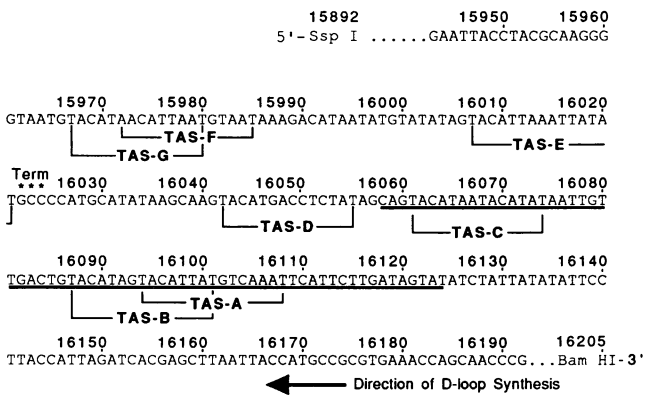

FIG. 2. Nucleotide sequence of the DNase I-protected region. A partial nucleotide sequence of the mtDNA insert of clone pBS314 is shown; the base pair numbering follows the convention of Anderson et al. (1). Except for an A-to-G transition at position 16079, the sequence of the 314-bp fragment is identical to that previously reported (1). The DNase I-protected region shown in Fig. 1 is underlined, and the locations of the D-loop strand 3′ termini are indicated (*). Seven sequence elements which share nucleotide similarities with the multispecies consensus TAS (21) are noted as TAS elements A through G.

represented a naturally occurring mitochondrial protein-mtDNA interaction, we used a DMS methylation protection assay on purified intact bovine mitochondria. While this assay has been used extensively to analyze protein-nuclear DNA interactions in vivo, its application to the mitochondrial organelle, to our knowledge, is novel. In two independent experiments, using brain cortex tissue from different animals, purified mitochondria were treated with DMS and assayed for methylation protection of the mtDNA. Both control and test mtDNAs were digested with *Bam*HI, which cleaves the mtDNA 182 bp upstream of the 3′ D-loop end at nucleotide 16205 and at two considerably more distant sites. This conveniently located restriction site provided a point of reference for localizing protein-mtDNA binding near the D-loop 3′ end. For both animals, a region of methylation protection which corresponded to a portion of the in vitro DNase I footprint was seen on each DNA strand (Fig. 3). Three G residues on the H strand (nucleotides 16089, 16096, and 16104) and two G residues on the L strand (nucleotides 16082 and 16093) were protected (Fig. 3A). One G residue (nucleotide 16084) as well as an A residue (nucleotide 16108) on the H strand were hypermethylated. Hypermethylation is thought to be the result of conformational changes in the DNA leading to an enhanced sensitivity to DMS (38). To confirm the results of Southern blot analysis, sites of methylation-mediated cleavage of L-strand mtDNA were also assayed by primer extension (Fig. 3B). A single G residue at position 16093 (marked with an asterisk) was clearly protected and a second at position 16082 was faintly protected, thus confirming the results of the Southern blot. The protected and hypermethylated sites were detectable in all mtDNA preparations originating from freshly prepared mitochondria; similarly purified mitochondria which were frozen prior to DMS treatment failed to yield reproducible results. This finding suggests that the carefully prepared mitochondria were sufficiently intact to reflect the in vivo status of mtDNA. This observation is not surprising, since analogous nuclear protein-DNA binding is not significantly disturbed during careful isolation of nuclei, as determined by comparative analyses with intact cells (37).

Contained within the in organello footprint region are two TAS-like elements (TAS-A and TAS-B) which were also protected in vitro. With the possible exception of two H-strand G residues (nucleotides 16144 and 16145), no other protected or hypermethylated sites were readily visible within the D-loop 3′-end region analyzed in Fig. 3A. The segment of mtDNA protected within the mitochondrion (nucleotides 16082 to 16108) was 27 bp in length, 40 bp shorter than the in vitro footprint (Fig. 1). The majority of the larger in vitro DNase I footprint extended from the region protected in organello forward toward the D-loop 3′ end.

Minimum mtDNA sequence requirements for mitochondrial protein binding. Because the DNase I footprinting technique is known to overestimate the size of the binding site, we used a methylation protection assay to more specifically compare in vitro protein-mtDNA binding with that found in the organelle. With use of the same protein fraction and labeled DNA fragment as for Fig. 1, as well as the methylation conditions used for Fig. 3, this procedure resulted in protected G residues at nucleotides 16082, 16084, 16089, 16093, 16096, and 16104 (Fig. 4). These sites of altered methylation reactivity correspond almost exactly to the in organello footprint. The only difference occurred at an H-strand G residue (nucleotide 16084) which was protected in vitro instead of hypermethylated and an H-strand A residue (nucleotide 16108) which did not exhibit hypermethylation. Several factors could account for differences between in vitro and in organello methylation reactivity, including mtDNA topological differences, different assay conditions, and potentially the selective loss of additional binding factors in the protein fraction used in vitro. More importantly, the fact that in vitro DMS protection so closely mimics the in organello situation demonstrates that TAS binding is not critically dependent on such differences and is comparable to that occurring within the organelle.

By testing a series of deleted DNAs in DNase I protection assays, we attempted to more precisely determine mtDNA sequences minimally required for protein binding. Initially, we used an *Rsa*I restriction site located at the 5′ end of TAS-A to subclone the mtDNA fragment spanning nucleotides 16092 to 16205. This fragment contains approximately half of the in organello protected region, including the TAS-A element and a small portion of TAS-B which overlaps into TAS-A. When this fragment was used in DNase I protection experiments, relatively strong binding was maintained on both DNA strands (Fig. 5). Protein-mtDNA interaction on the L strand began at position 16111, within three nucleotides of the distal end of the TAS-A element, and extended 14 bases into the vector DNA.

A 20-bp oligonucleotide (nucleotides 16092 to 16111), representing the minimum bovine L-strand mtDNA sequence protected in Fig. 5, was synthesized and cloned into pBluescript vector (clone pKS20). This fragment encompasses only TAS-A and five adjacent nucleotides (16092, 16093, 16109, 16110, and 16111) of the bovine sequence. As seen in Fig. 6, this 20-bp domain was sufficient for specific protein binding. Because the 20-bp target sequence encompasses solely TAS-A, it appears that only this element or some portion thereof is critical for protein recognition. It is interesting to note that in both Fig. 5 and 6, the DNase I footprint extended into vector sequences in the same directional manner relative to the TAS-A sequence. The dissimilarity of the protected vector sequences with each other and the positions of these sequences relative to the mtDNA insert suggest that protection of mtDNA sequences to the

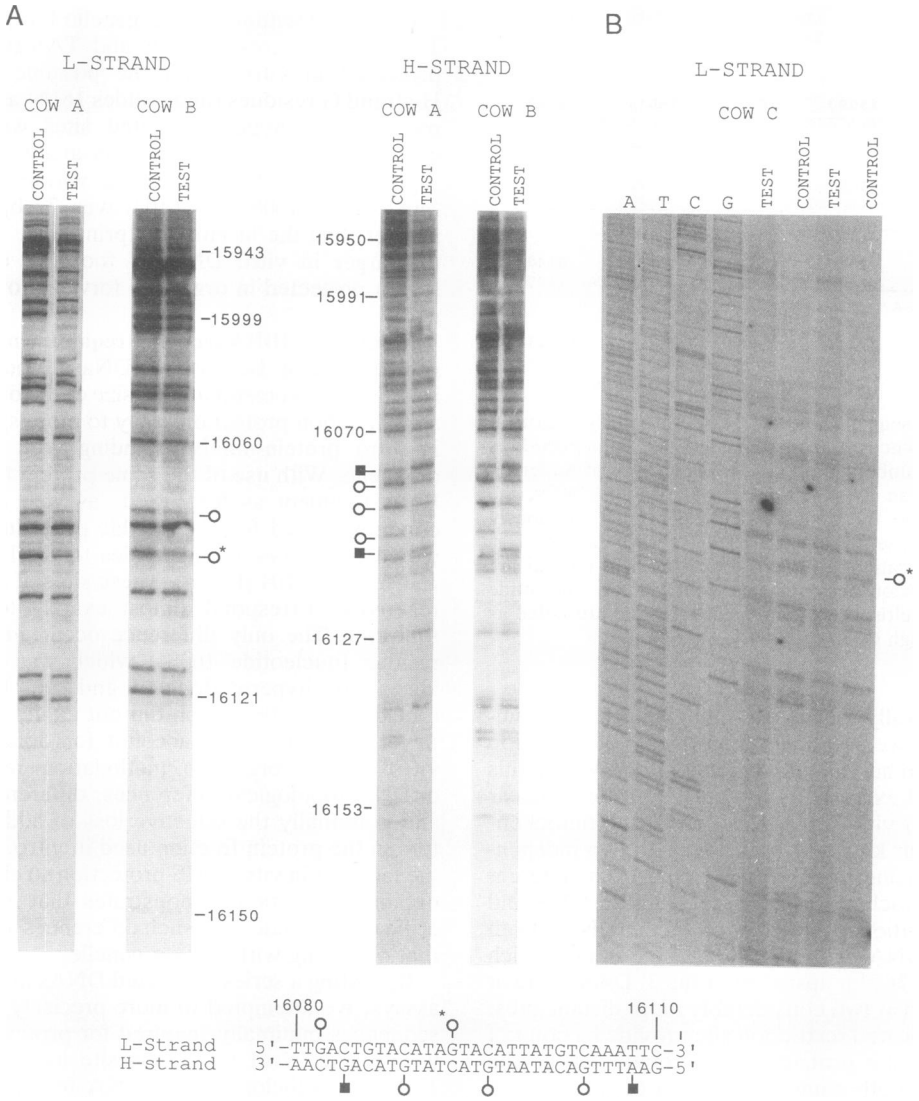

FIG. 3. In organello footprinting of the D-loop 3′-end region. Freshly purified bovine mitochondria were isolated from three individual animals (cows A through C) and treated with 0.5% DMS prior to mtDNA extraction and piperidine cleavage (test lanes). Naked mtDNA was treated with DMS to generate control G ladders (control lanes) for analyzing alterations of methylation patterns. (A) Purified mtDNA from cows A and B was digested with *Bam*HI at nucleotide 16205 (prior to piperidine cleavage) to provide a defined endpoint of reference, resolved on a sequencing gel, Southern blotted, and hybridized to RNA probes complementary to either L- or H-strand mtDNA. Sites of in organello protection and hypermethylation are marked with open circles and solid squares, respectively. (B) To confirm the L-strand Southern blot footprint, L-strand fragments from cow C were annealed to a labeled primer (WH202) and extended with *Taq* DNA polymerase for 10 cycles. A dideoxy sequencing reaction using the same primer and plasmid DNA (pBS314) was run alongside the extended products. The open circle with an asterisk denotes the same G residue (16093) footprinted in panel A. A sequence summary is given below the autoradiographs.

proximal side of TAS-A (the side closest to the D-loop 3′ end) in the larger intact mtDNA fragment is not sequence specific.

To determine whether protein binding could occur in the absence of TAS-A, we analyzed several cloned mtDNA fragments in which TAS-A was either entirely or partially absent. One fragment tested (nucleotides 16060 to 16100) contained all of TAS-C, TAS-B except for nucleotide 16101, and only the proximal half of TAS-A (nucleotides 16094 to 16100). In vitro footprint experiments using this fragment failed to demonstrate any apparent protection (data not shown). A cloned *Rsa*I fragment (nucleotides 16089 to 16060) containing only TAS-C and a cloned *Ssp*I-*Hin*cII fragment (nucleotides 15892 to 16079) containing TAS-C through TAS-G also failed to exhibit any apparent protection (data not shown). These observations support the conclusion that TAS-A is the principal protein recognition site in this part of the D-loop region. That the proximal half of TAS-A was insufficient for binding implies that critical bases for protein recognition reside in TAS-A-distal nucleotides 16101 to 16108.

Identification of a TAS-A-binding protein by UV cross-linking. A photochemical cross-linking protocol, previously used to identify the adenovirus major late transcription factor (9), was used here to identify a mitochondrial TAS-A-binding protein. A uniformly radiolabeled 171-bp DNA fragment, containing only the TAS-A 20-bp oligomer and adjacent vector sequences, was prepared by PCR amplifica-

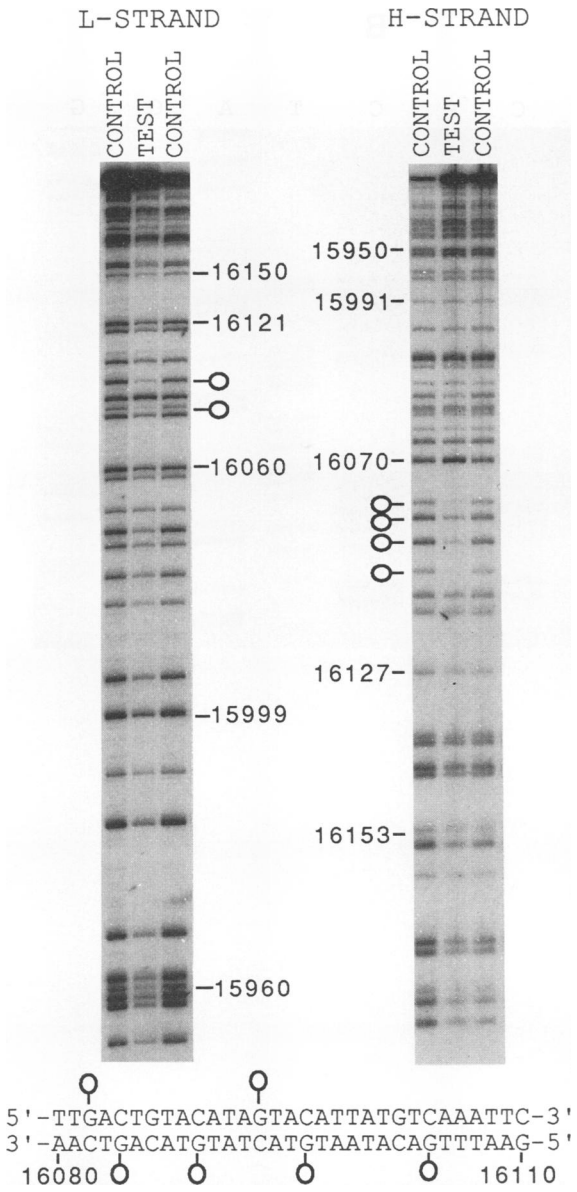

FIG. 4. In vitro identification of protein-mtDNA interaction in the D-loop 3'-end region, using methylation protection. Clone pBS314 containing the mtDNA 314-bp *Ssp*I-*Bam*HI fragment was end labeled on either the L strand or H strand, incubated with 30 ng of DEAE-Sephacel-purified mitochondrial protein fraction, and treated with DMS (test lanes). Control G ladders were generated by DMS treatment of the same fragment in the absence of protein (control lanes). Sites of methylation protection are indicated by open circles. A summary of protected sequences is given below the autoradiographs.

tion. This target sequence was then incubated in binding buffer with the same chromatographic protein fraction used in the in vitro footprint assays and exposed to a level of UV radiation sufficient to covalently cross-link DNA and associated protein. Prior to electrophoresis, the protein-DNA samples were extensively digested with nucleases to degrade all un-cross-linked sequences. In a UV irradiation time course experiment (Fig. 7A), a single major band migrating as a protein of approximately 48,000 molecular weight was visible after 5 min of irradiation. As expected, higher UV doses resulted in the appearance of additional labeled species. No bands were visible in the sample which did not undergo irradiation. Increased amounts of nuclease or time of digestion did not alter gel mobility of the 48-kDa species (data not shown).

To determine the specificity of protein binding in the UV cross-linking experiment, similar reactions were performed in the presence of various DNA competitors (Fig. 7B). Nonspecific competitor did not appreciably affect protein binding, while increasing amounts of a specific competitor containing the TAS-A sequence resulted in a corresponding decrease in signal intensity. As a final control, a uniformly radiolabeled probe lacking the TAS-A element was generated by PCR amplification of the parental pBluescript plasmid which contained no insert. UV cross-linking experiments using this probe failed to reveal a 48-kDa protein species. Since the attachment of short oligonucleotides does not significantly alter the mobility of proteins during SDS-polyacrylamide gel electrophoresis (9, 28), these experiments provide evidence that a mitochondrial protein of approximately 48 kDa binds specifically to the TAS-A sequence. The possibility that more than one 48-kDa species binds to TAS-A or that multiple proteins bind to TAS-A and in sum migrate as a 48-kDa species cannot, however, be formally eliminated.

DISCUSSION

We have identified a candidate bovine mitochondrial protein(s) that binds specifically to a sequence element which is evolutionally conserved among vertebrates (21). These conserved TAS elements are located near the 3′ end of the mitochondrial D loop, a region which is otherwise quite divergent (1, 42). Although mtDNA replication begins at the origin of H-strand synthesis, whether or not this initial synthesis event results in the production of an H-strand D-loop DNA or a complete mitochondrial genome is thought to be governed by a termination mechanism likely to involve TAS elements (12). By establishing in vitro and in organello that only one of several TAS-like domains functions as a specific protein recognition site, we provide evidence that D-loop termination is likely to be mediated through a highly sequence specific *trans*-acting factor.

The concept of a *trans*-acting factor regulating the efficiency of H-strand DNA termination may have far-reaching implications for understanding the regulation of mitochondrial activity. There are formally two ways in which a TAS-A-binding protein could regulate mtDNA replication. It could function as a negative regulator of H-strand elongation by promoting termination of DNA synthesis, thereby leading to D-loop formation. As a precedent for this type of mechanism, *Escherichia coli* leading-strand DNA synthesis is terminated in a sequence-specific manner in vitro through binding of TUS (termination utilization substance) protein factor (27). Alternately, TAS-A–protein binding could promote H-strand elongation by preventing termination at the D-loop 3′ end. In view of the fact that a footprint is seen in mitochondria from brain cortex tissue, it is reasonable to assume that a relatively high proportion of the TAS-A sites in the mitochondria are occupied by protein. High TAS occupancy in conjunction with rapid D-loop turnover (5) and abundance of D-loop mitochondrial forms in highly oxidative tissues (3) favor a mechanism in which protein binding to TAS leads to the early termination of H-strand replication and creation of D-loop strands; that is, these data favor the view that TAS-A binding is a negative regulator of H-strand

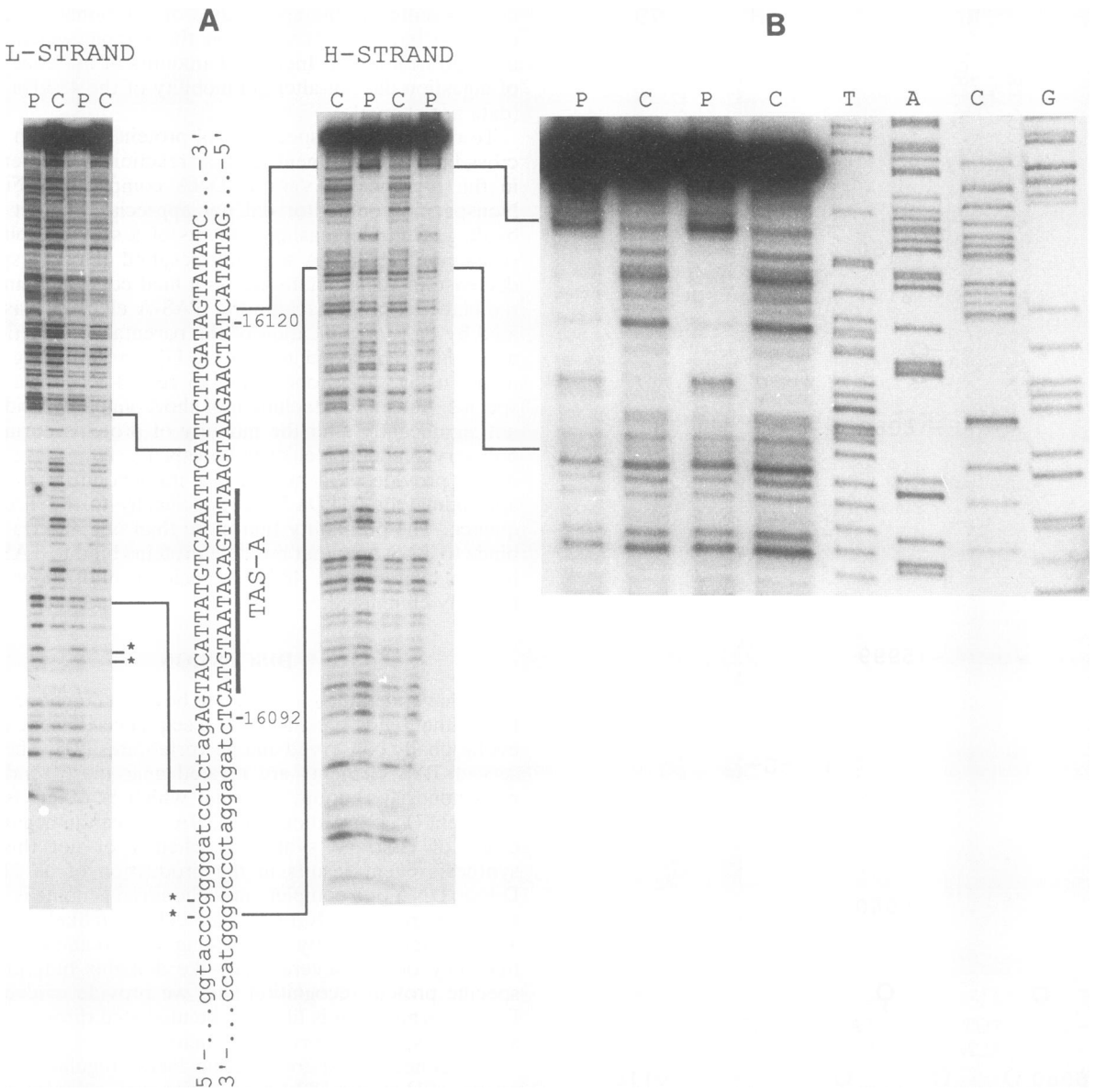

FIG. 5. (A) DNase I protection of TAS-A and adjacent D-loop 3'-proximal nucleotides. An mtDNA fragment encompassing nucleotides 16092 to 16205 (capital letters) was subcloned from pBS314, end labeled on either the L strand or H strand, incubated with 30 ng of DEAE-Sephacel-purified mitochondrial protein fraction, and digested with DNase I (lanes P). Control reactions (lanes C) were performed in a similar manner, omitting the addition of protein. (B) Expanded view of the H-strand footprint along with a sequencing ladder of known nucleotide composition as a size marker. Brackets indicate the protected region along with the actual nucleotide sequence of the labeled fragment. Asterisks indicate DNase I-hypersensitive sites. Lowercase letters indicate vector DNA.

mtDNA synthesis by causing termination of H-strand synthesis.

Conflicting observations regarding the abundance of the D-loop (triplex) mitochondrial forms and cellular and mitochondrial biogenesis suggest that a strict two-state model (bound TAS/unbound TAS) for regulating mtDNA replication is likely to be an oversimplification of what actually occurs in vivo. This becomes apparent when attempts are made to correlate D-loop frequency with intracellular mitochondrial replicative and transcriptional activities. For example, in a comparison of muscle subtypes from several mammalian species, the fraction of D-loop forms was shown to correlate directly with cellular respiratory capacity and other markers reflecting mitochondrial activity (3). The proportion of D loops in mitochondrial genomes has also been shown to increase during *X. laevis* oocyte maturation (7) and during mouse L-cell proliferation (32), situations requiring both active replication and active transcription. However, in contrast, studies have also reported that D-loop frequency decreased during active mitochondrial biogenesis. For example, analysis of regenerating hepatocytes in rats following partial hepatectomy demonstrated that the proportion of D-loop forms decreased relative to nonproliferating cells (24). Also in rats, upon aortic banding (a procedure which causes hypertrophy of the cardiac muscle and stimulates mitochondrial biogenesis), a significant decrease in

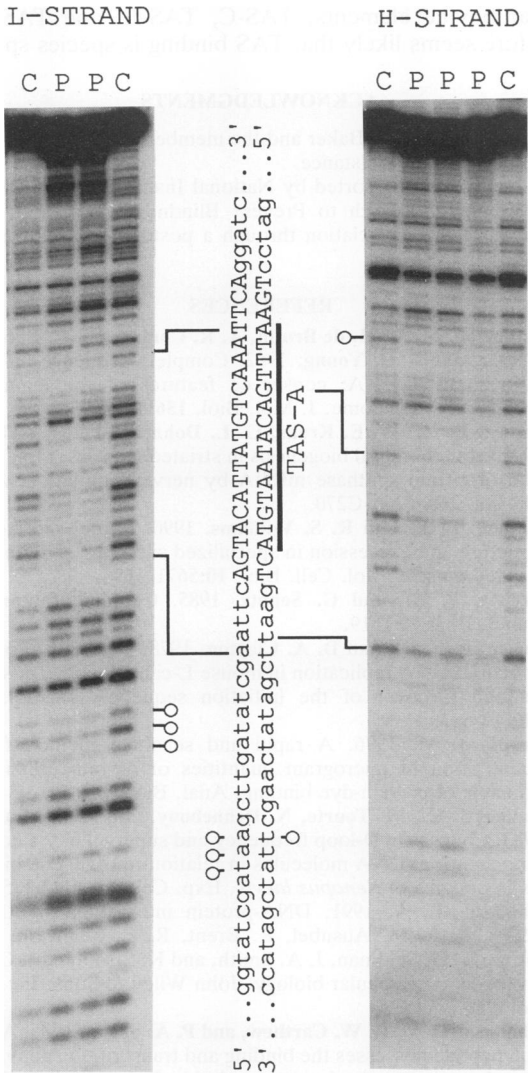

FIG. 6. DNase I protection of TAS-A. Complementary oligonucleotides corresponding to the L and H strands of mtDNA sequences 16092 to 16111 (capital letters) were synthesized with 5′ *Bam*HI-3′ *Eco*RI linkers and cloned into the corresponding polylinker sites of the pBluescript vector (lowercase letters). DNase I reactions were performed as described for Fig. 5. A sequencing ladder of known nucleotide composition was run alongside the samples as a molecular weight marker (not shown). Brackets indicate the protected region along with the actual nucleotide sequence of the labeled fragment. Open circles indicate additional vector-borne individual nucleotides protected from DNase I digestion.

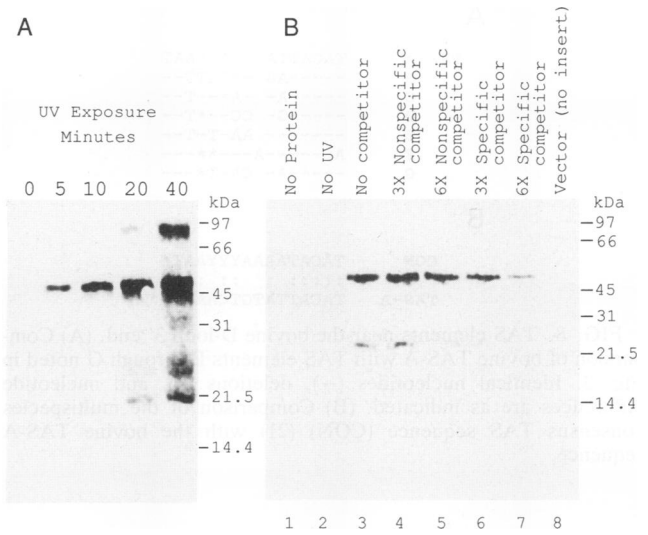

FIG. 7. Identification of TAS-A-binding protein by UV cross-linking. (A) Time course of cross-linking. Binding reaction mixtures contained 80 ng of the DEAE-Sephacel protein fraction used in the DNase I experiments and 1 ng of uniformly labeled probe containing TAS-A. Samples were irradiated for the indicated times and processed as described in Materials and Methods. Molecular weight markers are indicated. (B) Specificity of TAS-A binding. Binding reactions contained uniformly labeled probe (1 ng) either containing TAS-A (lanes 1 to 7) or plasmid vector sequences only (lane 8) and 80 ng of the DEAE-Sephacel protein fraction used in the DNase I experiments (lanes 2 to 8) or no protein (lane 1). Except for lane 2, all reactions were irradiated for 10 min. All reactions were processed as described above. Lanes: 1, no protein; 2, no irradiation; 3, probe alone; 4 and 5, three- and sixfold molar excess of plasmid DNA fragment, respectively; 6 and 7, three- and sixfold molar excess of plasmid DNA fragment containing TAS-A, respectively; 8, uniformly labeled plasmid DNA fragment.

D-loop frequency in heart muscle mitochondria was noted (40). Clearly, care must be taken when one attempts to relate mtDNA structure to synthetic activities among different cell types under different physiological conditions (3, 39). A more accurate model for regulating mtDNA replication is likely to involve not only TAS-binding protein but other factors responsible for initiation of H-strand synthesis. As previously suggested (13), two such candidate regulatory factors are mitochondrial transcription factor 1 (mtTF1), which enhances transcription at the L- and H-strand promoters, and mitochondrial RNA-processing enzyme, which cleaves transcripts from the L-strand promoter to presumably generate primers for H-strand synthesis. Synthesis of a complete mitochondrial genome may, in fact, require both an increased level of such initiation/priming factor(s) and a correspondingly decreased level of TAS-binding protein. However, under certain conditions, such as actively dividing mammalian cells in culture, in which initiation of H-strand DNA synthesis appears to be almost constitutive (5), producing a complete mitochondrial genome may be predominately regulated by the presence or absence of TAS binding.

Presently, there are two mammalian mitochondrial proteins which are known to bind specific mtDNA sequences: a ~34-kDa mitochondrial transcription termination factor (mTERF) (26) and mtTF1, which has a molecular weight of 24 kDa (18). Because mtTF1 binds mtDNA as a dimer of 48 kDa (13a), the size of the DNA-binding protein identified in this study, it seems possible that mtTF1 may be responsible for TAS-A binding activity. However, several observations suggest that this is not the case. The protein identified in this study appears to be highly specific for the TAS-A sequence. Titration of binding activity to levels severalfold greater than necessary for strong protection of TAS-A does not lead to protection at any of the other TAS-like sequences (Fig. 1 and data not shown). In contrast, mtTF1 shows an inherent flexibility in primary DNA sequence recognition requirements in several mammalian species (19, 20). Furthermore, no 24-kDa species (the size of an mtTF1 monomer) was evident in the UV cross-linking experiments (Fig. 7). Typically, if a protein binds to its target site as a dimer, UV

A

```
TAS-A     TACATTA TGTCAAAT
   B      -----AG -ACATT--
   C      -----A- -A*--T--
   D      -----G- CC--*T--
   E      ------- AA-T-T--
   F      A------A---**---
   G      -----A- CA-T*---
```

B

```
CON       TACATAAAAYYYAAT
          ::::: :  :: :::
TAS-A     TACATTATGTCAAAT
```

FIG. 8. TAS elements near the bovine D-loop 3′ end. (A) Comparison of bovine TAS-A with TAS elements B through G noted in Fig. 2. Identical nucleotides (−), deletions (*), and nucleotide differences are as indicated. (B) Comparison of the multispecies consensus TAS sequence (CON) (21) with the bovine TAS-A sequence.

cross-linking (as described in Materials and Methods) results in the transfer of label to only one of the two bound molecules and the observed molecular weight is likely to be that of a monomer (8). Therefore, current data favor TAS-A-binding protein being a single species of about 48 kDa, unrelated to mtTF1.

Since all open reading frames present in the mitochondrial genome have been characterized (10), the 48-kDa protein which binds TAS-A must represent a nuclear-encoded gene product. Time course studies on nuclear and mitochondrial gene induction in response to enhanced contractile activity in rabbit muscle indicate that neural input is initially sensed in the nucleus and that expression of mitochondrial genes is regulated as a secondary event (2). Furthermore, enhancement of mitochondrial gene expression appears to be the result of increased mitochondrial gene dosage levels and not enhanced rates of transcription per genome (3). Therefore, proteins like a nuclear-encoded TAS-binding protein may represent an important nuclear-mitochondrial link for regulating cellular respiratory capacity. Again, however, care must be taken in drawing simple conclusions since there is one clear example during *X. laevis* embryonic development in which up-regulation of mitochondrial transcription precedes induction of mtDNA replication (16). Thus, balances between mtDNA copy number and rates of replication versus transcription are likely to be regulated by multiple factors, TAS-binding protein being but one.

Additional insight regarding the sequence requirements for TAS-A binding can be gained from a comparison of TAS-A with the other TAS-like elements which do not support binding. As shown in Fig. 8A, no other TAS-like element is identical to TAS-A, with almost all sequence dissimilarity clustered between eight nucleotides (16099 and 16106, TATGTCAA). Therefore, since these nucleotides uniquely define TAS-A as the only protected element in this region, some or all must be critical for protein recognition. This conclusion is consistent with the observation that nucleotides 16094 to 16100 alone are not sufficient for protein binding (see Results). Comparison of TAS-A with the TAS consensus sequence, previously derived from 11 species (21), shows that the basic TAS motif has been maintained (Fig. 8B). However, among the many candidate TAS elements identified in several mammalian species, including those which reportedly have only one TAS element such as the human (14), there is a greater range of sequence dissimilarity than is exhibited between bovine TAS-A and its most similar bovine elements, TAS-C, TAS-E, and TAS-F. It therefore seems likely that TAS binding is species specific.

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