Borovjagin, V. L., Vergara, J. A., & McIntosh, T. J. (1982) *J. Membr. Biol. 69*, 199–212.
Casal, H. L., & Mantsch, H. H. (1984) *Biochim. Biophys. Acta 779*, 381–401.
Chattopadhyay, A., & London, E. (1986) *Biophys. J. 49*, 308a.
Cheng, K.-H., Yeagle, P. L., Lepock, J. R., & Hui, S. W. (1986) *Biophys. J. 49*, 324a.
Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
De Kruijff, B., Verkleij, A. J., Leunissen-Bijvelt, J., van Echteld, C. J. A., Hille, J., & Rijnbout, H. (1982) *Biochim. Biophys. Acta 693*, 1–12.
Düzgüneş, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., & Papahadjopoulos, D. (1985) *Biochemistry 24*, 3091–3098.
Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.
Ellens, H., Bentz, J., & Szoka, F. C. (1986) *Biochemistry 25*, 285–294.
Epand, R. M. (1985) *Biochemistry 24*, 7092–7095.
Faucon, J.-F., & Lussan, C. (1973) *Biochim. Biophys. Acta 307*, 459–466.
Harlos, K., & Eibl, H. (1981) *Biochemistry 20*, 2858–2892.
Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) Vol. 1, pp 71–123, Academic, London.
Madden, T. D., & Cullis, P. R. (1982) *Biochim. Biophys. Acta 684*, 149–153.
Rand, R. P., & Sengupta, S. (1972) *Biochim. Biophys. Acta 255*, 484–492.
Seddon, J. M., Cevc, G., & Marsh, D. (1983) *Biochemistry 22*, 1280–1289.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta 515*, 367–394.
Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1977) *Biochemistry 16*, 819–828.
Struck, D., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.
Vasilenko, I., De Kruijff, B., & Verkleij, A. J. (1982) *Biochim. Biophys. Acta 684*, 282–286.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43–63.
Waggoner, A. S., & Stryer, L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 579–589.
Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.

Isolation and Characterization of a Complementary DNA for the Nuclear-Coded Precursor of the β-Subunit of Bovine Mitochondrial F_1-ATPase†

Gail A. M. Breen,* Priscilla L. Holmans, and Karen E. Garnett

Molecular and Cell Biology Program, The University of Texas at Dallas, P.O. Box 830688, Richardson, Texas 75083-0688

Received October 8, 1987; Revised Manuscript Received January 29, 1988

ABSTRACT: We have isolated a cDNA clone encoding the precursor of the β-subunit of the bovine heart mitochondrial F_1-ATPase. Two probes were used to isolate this precursor from a bovine heart cDNA library. One probe was a mixed-sequence oligonucleotide directed against a portion of the amino acid sequence of the mature protein, and the other probe was the F_1-ATPase β-subunit gene from *Saccharomyces cerevisiae*. Determination of the nucleotide sequence of this cDNA reveals that it contains a 1584-nucleotide-long open reading frame that encodes the complete mature β-subunit protein and a 48 amino acid long NH_2-terminal extension. This amino-terminal presequence contains four basic arginine residues, one acidic glutamic acid residue, four polar uncharged serine residues, and five proline residues. Southern blot hybridization analyses suggest that the bovine F_1-ATPase β-subunit precursor is encoded by a single genetic locus. RNA blot hybridization analyses reveal a single mRNA species of approximately 1.9 kilobases from both bovine liver and heart.

The mitochondrial ATP synthase complex synthesizes ATP from ADP and P_i, utilizing energy from the electron-transport chain. This complex consists of two distinct regions: a soluble F_1 region that contains the catalytic site for ATP synthesis and a membrane-integrated F_0 region that is involved in proton translocation [for reviews, see Amzel and Pedersen (1983), Futai and Kanazawa (1983), and Senior and Wise (1983)]. The ATP synthase complex, like several of the other complexes of the mitochondrial oxidative phosphorylation system, is composed of proteins encoded by two distinct genetic systems. For example, two of the subunits of the F_0 region of the mammalian ATP synthase complex are encoded by the mitochondrial DNA and translated on ribosomes within the mitochondria (Anderson et al., 1981; Bibb et al., 1981; Fearnley & Walker, 1986). The rest of the subunits are encoded by nuclear genes, synthesized on cytoplasmic ribosomes (often as larger precursors), imported into mitochondria, processed to give the mature subunits, and then assembled together with the other subunits to form a functional complex [for reviews, see Tzagoloff and Myers (1986) and Douglas et al. (1986)]. The molecular mechanisms that govern the expression of the nuclear genes that encode proteins of the mitochondrial oxidative phosphorylation system and their coordination with the mitochondrial genes are largely unknown. Furthermore, the sequences required for the import and sorting of these proteins to the mitochondrion have not

† This work was supported by a grant from the National Institutes of Health.
* Address correspondence to this author.

0006-2960/88/0427-3955$01.50/0

yet been fully elucidated. The molecular cloning of these genes is a first step in investigating these problems. As a part of studies involving the isolation and characterization of nuclear genes encoding subunits of the bovine ATP synthase complex, we have isolated a cDNA encoding the precursor of the β-catalytic subunit of this complex. Two probes were used to isolate this cDNA. One was a mixture of 64 oligonucleotides, 17 bases long, with sequences predicted from a segment of the known amino acid sequence of the mature protein (Runswick & Walker, 1983). The other was the cloned β-subunit gene from *Saccharomyces cerevisiae* (Takeda et al., 1985). Determination of the nucleotide sequence of this cDNA reveals that it contains the complete coding region of the mature β-subunit protein and a 48 amino acid long NH_2-terminal presequence. This cDNA hybridized to a single mRNA species, approximately 1.9 kilobases (kb) in length, from both bovine liver and heart. Southern blot hybridization analyses suggest that the F_1-ATPase β-subunit is present as a single copy gene in the bovine genome.

Experimental Procedures

Oligonucleotide Synthesis. A mixed-sequence oligonucleotide was synthesized by the solid-phase phosphotriester method (Gait et al., 1982) (Figure 1). This oligonucleotide corresponds to amino acid residues 291–296 of the mature bovine F_1-ATPase β-subunit (Walker et al., 1985; Runswick & Walker, 1983). The mixture contains 64 discrete sequences.

Library Screening. A bovine heart cDNA library constructed in the plasmid vector pBR322 was kindly provided by Dr. S. McKnight (Uhler et al., 1986). Transformed cells were plated in duplicate at a density of approximately 5000 colonies per 82-mm-diameter nitrocellulose filter on LB agar plates containing 10 μg of tetracycline/mL and then amplified on LB agar plates containing 150 μg of chloramphenicol/mL. Colonies were screened (Hanahan & Meselsen, 1980) using a nick-translated (Rigby et al., 1977) 0.9-kb *Bam*HI–*Pvu*II fragment of the F_1-ATPase β-subunit gene of *S. cerevisiae* (Takeda et al., 1985). Hybridization was carried out at 30 °C for 24 h in a solution containing 10× Denhardt's [1× = 0.02% poly(vinylpyrrolidone)/0.02% Ficoll/0.02% bovine serum albumin], 50% formamide, 6× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 0.5% NP-40, 0.1% sodium dodecyl sulfate (SDS), 100 μg/mL salmon sperm DNA, and 10^6 cpm/mL ^{32}P-labeled probe. Following hybridization, filters were washed at 40 °C in 0.2× SSC/0.1% SDS and then exposed to X-ray film at −70 °C with intensifying screens. Transformants were also screened (Suggs et al., 1981) by using a mixed-sequence oligonucleotide directed against a portion of the amino acid sequence of the mature bovine F_1-ATPase β-subunit (Figure 1). The oligonucleotide was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified by Sephadex G-25 chromatography (Maniatis et al., 1982). Hybridization was carried out at 37 °C using 7×10^5 cpm/mL ^{32}P-labeled oligonucleotide in a solution containing 6× SSC, 1× Denhardt's, 0.1% SDS, and 20 μg/mL yeast tRNA. Following hybridization, filters were washed in 6× SSC/0.1% SDS at 47 °C and then exposed to X-ray film at −70 °C with intensifying screens. Colonies that hybridized on duplicate filters were isolated and rescreened.

Large-Scale Plasmid Preparation. Plasmid DNAs suitable for sequencing were isolated by centrifugation in CsCl/ethidium bromide gradients after lysis of the bacterial cells as described (Guerry et al., 1973).

DNA Sequence Analysis. DNA was sequenced by using either the chemical modification (Maxam & Gilbert, 1980) or the dideoxy chain-termination method (Sanger et al., 1977). For the chemical modification method, restriction endonuclease fragments were sequenced after radiolabeling at their 5′ termini using T4 polynucleotide kinase and [γ-^{32}P]ATP or at their 3′ termini using the Klenow fragment of *Escherichia coli* DNA polymerase I and the appropriate [α-^{32}P]dNTP. For the dideoxy chain-termination method, DNA restriction fragments were first subcloned into the plasmid vector pUC18 and sequenced after denaturation of the plasmid DNA with alkali as described (Chen & Seeburg, 1985).

Northern RNA Blot Analysis. Total cellular RNA was prepared from tissues by homogenization in guanidinium thiocyanate, followed by centrifugation through a cesium chloride cushion (Chirgwin et al., 1979). Poly(A^+)-containing RNA was isolated from total RNA by chromatography on oligo(dT)–cellulose (Maniatis et al., 1982). Total RNA or poly(A^+) RNA was denatured with 50% formamide and 2.2 M formaldehyde at 65 °C and then separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (Maniatis et al., 1982). The separated RNA species were transferred to nitrocellulose (Thomas, 1980) and hybridized with a ^{32}P-labeled fragment of the β-subunit cDNA. The 1.2-kb *Pst*I restriction fragment of the cDNA was labeled with ^{32}P to a specific activity of 10^9 cpm/μg using the random-primer method (Feinberg & Vogelstein, 1983). Hybridization was at 42 °C for 24 h in a solution containing 50% formamide, 6× SSC, 5× Denhardt's, 50 mM NaH_2PO_4 (pH 6.5), 100 μg/mL yeast tRNA, and 10^6 cpm/mL ^{32}P-labeled probe. Following hybridization, the filters were washed at 65 °C in 0.1× SSC/0.1% SDS and then exposed to X-ray film at −70 °C with intensifying screens.

Southern Analysis of Genomic DNA. Ten micrograms of calf thymus DNA (Worthington, Freehold, NJ) was digested with various restriction enzymes, separated by electrophoresis in 0.7% agarose gels, and transferred to nitrocellulose filters (Southern, 1975). The filters were probed with a ^{32}P-labeled 340 base pair (bp) *Pst*I–*Nco*I fragment of the F_1-ATPase β-subunit cDNA. Hybridization was carried out for 48 h at 42 °C in a solution containing 50% formamide, 6× SSC, 5× Denhardt's solution, 50 mM NaH_2PO_4 (pH 6.5), 100 μg/mL yeast tRNA, and 10^6 cpm/mL ^{32}P-labeled probe. Nitrocellulose filters were washed at 65 °C in 0.1× SSC/0.1% SDS and then exposed to X-ray film at −70 °C with intensifying screens.

Results

Isolation of cDNA Clones Homologous to the β-Subunit of the Mitochondrial F_1-ATPase. Two strategies were used to obtain bovine heart cDNA clones that are homologous to the β-subunit of the mitochondrial F_1-ATPase. First, we screened a bovine heart cDNA library constructed in the plasmid vector pBR322 using the β-subunit gene from *S. cerevisiae* as a probe. Sequence data have revealed that the yeast and bovine F_1-ATPase β-subunits are highly homologous, approximately 75% at the amino acid level (Takeda et al., 1985). Screening with the yeast probe under low stringency conditions identified eight clones. Four of these clones also hybridized to a 17-base-long mixed-sequence oligonucleotide containing all possible codons for a portion of the known amino acid sequence of the mature bovine β-subunit (Runswick & Walker, 1983) (Figure 1). Southern analyses of DNA isolated from these four clones revealed inserts ranging in size from approximately 600 to 1900 bp. Restriction mapping analyses showed that the cDNA inserts had the same cleavage maps with one another in their overlapping region. One of these clones (B42) had the longest insert, approximately 1.9 kilobase pairs (kbp) in length.

		291					296	
β-ATPase		MET	GLY	THR	MET	GLN	GLU	
mRNA	5'	AUG	GGN	ACN	AUG	CA A/G	GA A/G	3'
DNA probe	3'	TAC	CCN	TGN	TAC	GT T/C	CT	5'

FIGURE 1: Sequence of synthetic oligonucleotide mixture used as a probe in screening cDNA clones for β-subunit mRNA. Shown are the amino acid sequence of residues 291–296 of the mature bovine F_1-ATPase β-subunit (top line), the corresponding nucleotides (middle line), and all possible complementary sequences of the 17-base-long oligonucleotides that were synthesized for use as a probe (bottom line).

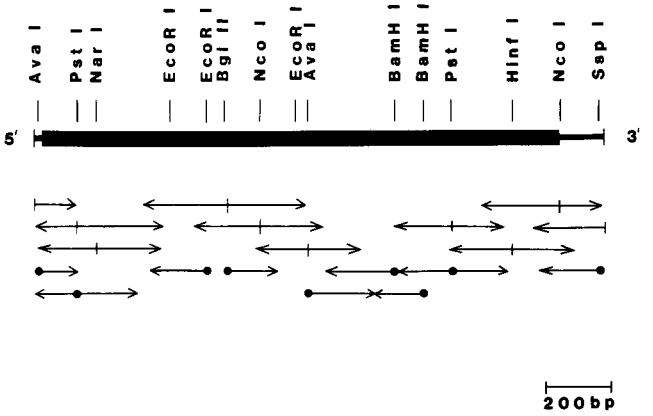

FIGURE 2: Restriction map of the bovine F_1-ATPase β-subunit cDNA and the strategy used for nucleotide sequence determination. The protein coding region is indicated by the closed box. Arrows represent the direction and the length of sequence determined for each independent experiment. Solid circles at the ends of the arrows indicate sequences determined by the dideoxy chain-termination method; vertical lines at the ends of arrows indicate sequences determined by the chemical degradation procedure.

Sequence Analysis of the cDNA Clone B42. The complete nucleotide sequence of the insert from the positive clone, B42, was determined on both strands by using the strategy shown in Figure 2. The nucleotide sequence of this cDNA and the deduced amino acid sequence are shown in Figure 3. Analysis of this nucleotide sequence predicts an open reading frame beginning at an ATG codon and extending for 1584 nucleotides before terminating at a TGA stop codon. This open reading frame is flanked by 19 bp of noncoding DNA at the 5′ end and 244 bp of noncoding DNA at the 3′ end (Figure 3). The 3′ noncoding sequence contains the consensus polyadenylation signal sequence AATAAA (Proudfoot & Brownlee, 1976), 14 nucleotides upstream from an 82-base-long poly(dA) tail. Thus, the 3′ end of the mRNA is represented in the cDNA. The protein encoded by this open reading frame is 528 amino acids in length and has a calculated molecular weight of 56 276. Analysis of the sequence of this protein reveals that it contains all of the 480 amino acid long sequence of the mature β-subunit protein. The amino acid sequence of the mature β-subunit, predicted from the nucleotide sequence of this cDNA clone, differs from that obtained by direct amino acid analysis (Runswick & Walker, 1983) at eight positions. Differences were observed at residue 134 where the cDNA specifies an isoleucine residue instead of a leucine residue, at position 139 an isoleucine instead of an aspartic acid, at position 167 a leucine instead of phenylalanine, at residue 226 a glutamic acid instead of glutamine, at residue 290 an aspartic acid instead of asparagine, at position 326 a threonine instead of valine, at position 357 a serine instead of a glycine, and at residue 361 an aspartic acid instead of an asparagine (Figure 3). This cDNA also contains 173 bp preceding the sequence that encodes the mature β-subunit protein. These 173 bp contain an open reading frame in phase with the mature β-subunit protein, with a methionine residue at amino acid −48. Examination of the 48 residues comprising this amino-terminal presequence reveals that it contains 4 basic arginine residues, 1 acidic aspartic acid residue, and 4 polar uncharged serine residues. Interestingly, this presequence also contains 5 proline residues. The calculated molecular mass of this putative leader sequence is approximately 4750 daltons.

RNA Analysis. The cDNA insert in B42 was isolated and used as a probe to examine the expression of the F_1-ATPase β-subunit precursor gene transcripts in bovine heart and liver. RNA blot hybridization analyses revealed a single hybridizing species of approximately 1.9 kb in both tissues (Figure 4). The β-subunit precursor was approximately 10-fold more abundant in bovine heart than in bovine liver. From the size of these RNA transcripts, it appears that the cDNA insert in B42 is nearly full-length.

Analysis of F_1-ATPase β-Subunit Genomic Sequences. Bovine genomic DNA was digested with various restriction enzymes; the fragments were separated by electrophoresis and examined by Southern blot hybridization using fragments of the β-subunit cDNA. Cleavage of the bovine genomic DNA with restriction enzymes that are predicted to give one band from the sequence of the cDNA gave a unique band when probed with a 340 bp *Pst*I–*Nco*I fragment of the β-subunit cDNA, suggesting that the F_1-ATPase β-subunit is present as a single copy gene in the bovine genome (Figure 5). Analyses of similar Southern blots with other fragments of the B42 cDNA insert revealed multiple bands, suggesting the presence of introns within regions coding for the F_1-ATPase β-subunit.

DISCUSSION

In this paper, we report the isolation of a cDNA clone encoding the precursor of the β-subunit of the bovine mitochondrial F_1-ATPase complex. Two probes were used to isolate this precursor from a bovine heart cDNA library (Uhler et al., 1986). One probe was a mixed-sequence oligonucleotide directed against a portion of the amino acid sequence of the mature β-subunit protein (Runswick & Walker, 1983), and the other probe was the cloned β-subunit gene (*atp2*) from *S. cerevisiae* (Takeda et al., 1985). Thus, cDNAs encoding subunits of the mammalian ATP synthase complex can be isolated by using either oligonucleotide probes for those subunits whose amino acid sequences have been determined (Walker et al., 1985) or heterologous probes for those subunits that are highly conserved. A third strategy was used in another laboratory to isolate a cDNA encoding the human F_1-ATPase β-subunit precursor, that of screening a human cDNA library in an expression vector using an antiserum generated against the yeast β-subunit protein (Ohta & Kagawa, 1986).

Analysis of the sequence of the longest cDNA indicates that the bovine heart F_1-ATPase β-subunit is initially synthesized with an amino-terminal extension at least 48 amino acids in length. We have assigned the translation initiation site to an in-frame ATG codon 144 bases upstream from the CAA codon which encodes glutamine, the first amino acid of the mature bovine β-subunit (Walker et al., 1985). It is likely that this ATG is the initiator methionine since the nucleotide sequence around this codon agrees with the consensus eucaryotic translational initiation site, $CC^{A}_{G}CCAUG$ (Kozak, 1984; Lutcke et al., 1987). However, since there is no termination codon upstream from this ATG in the cDNA clone, we cannot rule out the possibility that there is an additional upstream methionine.

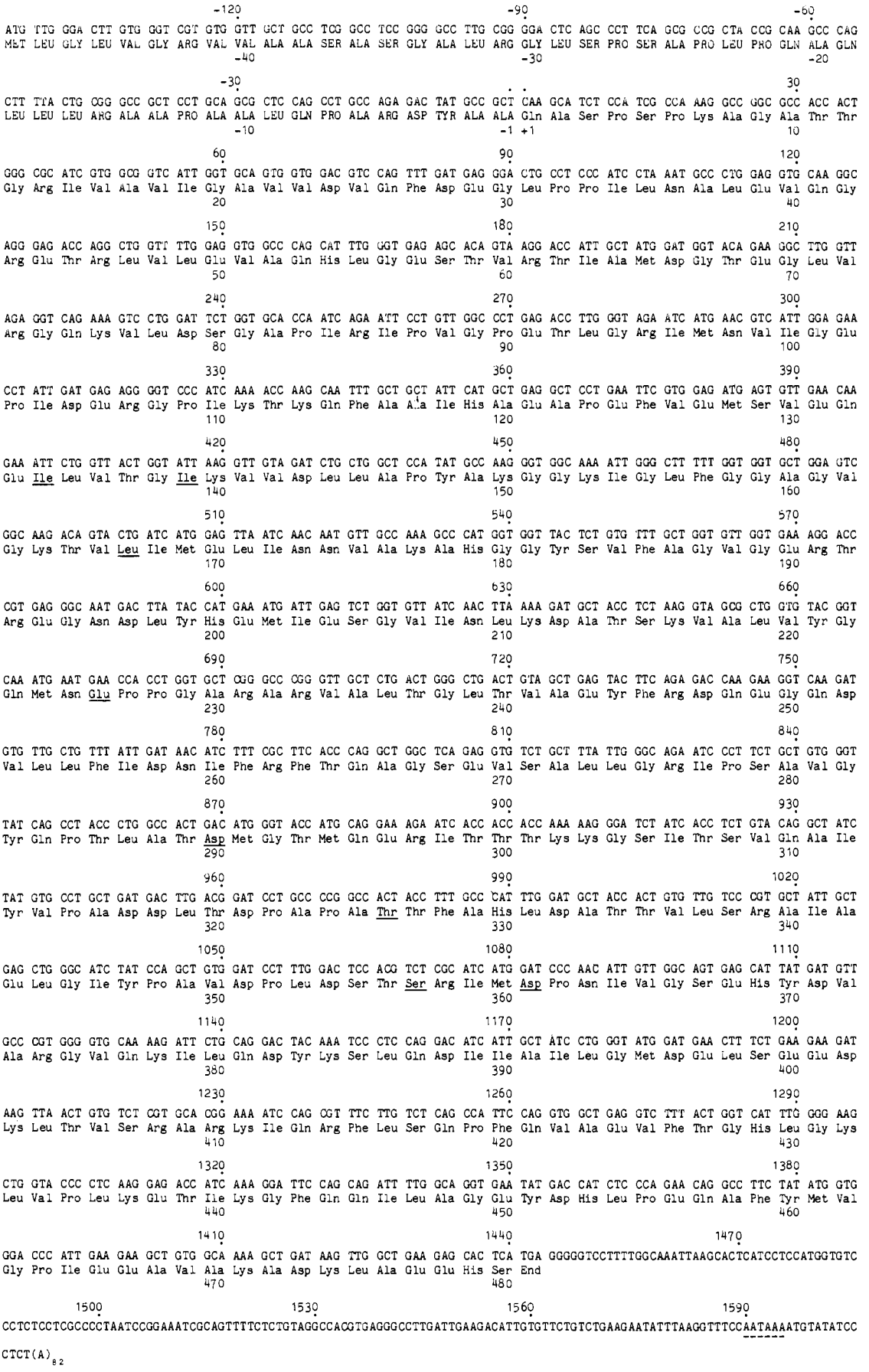

FIGURE 3: Nucleotide and predicted amino acid sequences of the bovine F_1-ATPase β-subunit precursor. Nucleotides and amino acids are numbered by using the following conventions. The glutamine residue found at the amino terminus of the mature β-subunit and its first corresponding base (C) are designated +1. All downstream amino acids and nucleotides carry (+) signs and all upstream amino acids and nucleotides (−) signs. The predicted amino acid residues that differ from those determined by direct amino acid sequence analysis are underlined. The consensus polyadenylation signal sequence, AATAAA, is underlined with a dotted line.

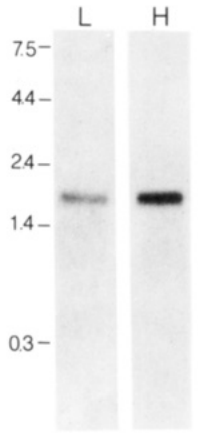

FIGURE 4: Northern blot hybridization analysis of the F_1-ATPase β-subunit precursor mRNA. Bovine liver (L) mRNA, 0.45 μg, and heart (H) mRNA, 0.075 μg, were denatured, separated by electrophoresis in 1.2% agarose gels in the presence of 2.2 M formaldehyde, and then transferred to nitrocellulose filters. The filters were hybridized with a ^{32}P-labeled probe derived from the bovine β-subunit cDNA. Size markers are indicated in kilobases.

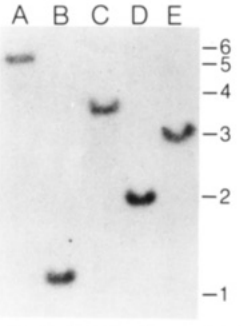

FIGURE 5: Southern blot hybridization analysis of bovine genomic DNA. Bovine genomic DNA (10 μg) was digested with restriction endonucleases: *Apa*I (lane A), *Bam*HI (lane B), *Bgl*II (lane C), *Ssp*I (lane D), and *Taq*I (lane E). The fragments were separated by electrophoresis in 0.7% agarose gels and transferred to nitrocellulose filters as indicated under Experimental Procedures. The filters were hybridized to a ^{32}P-labeled probe derived from the β-subunit cDNA. Size markers are in kilobases.

The NH_2-terminal presequence of the bovine heart F_1-ATPase β-subunit has many of the features of other presequences which target proteins to the mitochondrion [for reviews, see Douglas et al. (1986) and Schatz (1987)]. These include a preponderance of nonpolar amino acid residues (e.g., alanine, valine, and leucine) and hydroxylated amino acid residues (e.g., serine) and an overall positive charge (the bovine β-subunit precursor has four positively charged arginine residues and one negatively charged aspartic acid residue). The presence of an acidic amino acid residue in a mitochondrial import sequence is rare but not unique; for example, an acidic residue is also present in the presequence of the human F_1-ATPase β-subunit (Ohta & Kagawa, 1986), the bovine mitochondrial proteolipids (Gay & Walker, 1985), the bovine cytochrome P-450 (SSC) enzyme (Morohashi et al., 1984), the bovine mitochondrial phosphate carrier protein (Runswick et al., 1987), and the yeast cytochrome c_1 (Sadler et al., 1984). An unusual feature of the bovine F_1-ATPase β-subunit precursor is the presence of five proline residues. It has been proposed that mitochondrial presequences can form an amphiphilic α-helix structure that enables the presequence to interact directly with energized mitochondrial membranes (Roise et al., 1986; von Heijne, 1986). However, it is unlikely that the bovine F_1-ATPase β-subunit presequence will form such a structure due to the large number of proline residues that can introduce distortions in the helix (Terwilliger & Eisenberg, 1982; Sundaralingam et al., 1985).

The presequence of the β-subunit of the mitochondrial F_1-ATPase has now been determined for representatives of three kingdoms (animals, fungi, and plants), allowing an evolutionary comparison. Such a comparison reveals that there is little similarity in either the length or the primary structure of the β-subunit leader peptides among the members of the three kingdoms (see Figure 6). There is, however, a high degree of homology between the β-subunit leader peptides of the two animal species examined; the overall homology between the bovine (this work) and the human (Ohta & Kagawa, 1986) β-subunit leader peptides is 55% at the amino acid level. In addition to this overall homology, there are several regions and features of the β-subunit presequences that are conserved between the two animal species (Figure 6). For example, the four basic arginine residues and the one acidic glutamic acid residue found in the bovine β-subunit leader peptide are located at identical positions in the human leader peptide. In addition, both the bovine and human F_1-ATPase β-subunit leader peptides contain five proline residues, and three of these prolines are located at conserved positions in the two sequences. Interestingly, the amino acid sequence surrounding one of these conserved proline residues is somewhat homologous to the sequence of a region of the β-subunit leader peptide of *Nicotiana plumbaginifolia* (Boutry & Chua, 1985; see Figure 6). Similar domains which contain one or two proline residues and one or more positively charged amino acid residues have been proposed to play a role in the import of several proteins to the chloroplast (Schmidt & Mishkind, 1986; Karlin-Neumann & Tobin, 1986) and of subunit 9 of the ATP synthase complex of *Aspergillus nidulans* to the mitochondrion (Ward & Turner, 1986). These proline residues may contribute to a distinct secondary structure in the transit peptide, or they may facilitate a change in the conformation of the precursor as it crosses the mitochondrial membrane, since proline residues can undergo cis/trans isomerization during protein unfolding (Kim & Baldwin, 1982). However, if such regions of homology are important for import or processing, they are

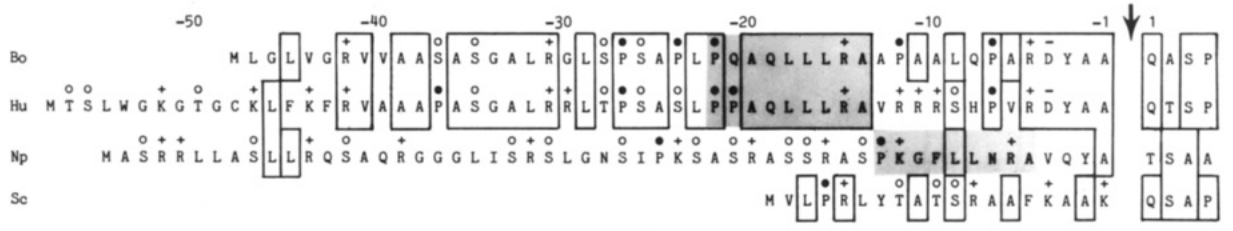

FIGURE 6: Comparison of the F_1-ATPase β-subunit presequences from bovine, human, *S. cerevisiae*, and *N. plumbaginifolia*. The F_1-ATPase β-subunit presequences from bovine (Bo; this work), human (Hu; Ohta & Kagawa, 1986), *S. cerevisiae* (Sc; Vasarrotti et al., 1987), and *N. plumbaginifolia* (Np; *atp2-1*; Boutry & Chua, 1985; Kobayashi et al., 1986) are aligned according to the putative NH_2-terminal amino acid residue of the mature protein (indicated by +1 after the arrow). Positively charged lysine and arginine residues are indicated by a (+), hydroxylated serine and threonine residues by a (O), negatively charged aspartic acid residues by a (−), and proline residues by a (●). The shaded regions indicate sequences that are homologous between the bovine, human, and *N. plumbaginifolia* β-subunit leader peptides.

required by only a subset of proteins since they are not found in all imported mitochondrial preproteins.

In contrast to the leader peptides of the β-subunit, the mature F_1-ATPase β-subunits are highly conserved among different species. For example, the amino acid sequence of the mature bovine β-subunit is 98% homologus to the human sequence (Ohta & Kagawa, 1986), 75% homologous to the *S. cerevisiae* sequence (Takeda et al., 1985), 70% homologous to the *N. plumbaginifolia* sequence (Boutry & Chua, 1985), and 70% homologous to the *E. coli* sequence (Walker et al., 1984). In spite of this sequence conservation, the codon usage of the β-subunit varies widely among the different species. For example, the most common codon for leucine is CTG in the bovine, human, and *E. coli* β-subunit, CTT and TTG in the *N. plumbaginifolia* mitochondrial β-subunit (*atp2-1*), and TTG in the *S. cerevisiae* β-subunit.

Our Southern blot hybridization data suggest that the F_1-ATPase β-subunit gene is encoded by a single genetic locus in the bovine genome. There is also one β-subunit gene in the genome of *S. cerevisiae* (Takeda et al., 1985). In contrast, in *N. plumbaginifolia*, there are two nuclear genes that encode two closely related F_1-ATPase β-subunit polypeptides (Boutry & Chua, 1985). The significance of two highly homologous β-subunits in *N. plumbaginifolia* is unclear. A single transcript, approximately 1.9 kb in length, was detected for the bovine F_1-ATPase β-subunit precursor in both liver and heart.

After this work was completed, a report was published describing the isolation and nucleotide sequence of a partial cDNA for the mature bovine F_1-ATPase β-subunit (Wallace et al., 1987). A comparison of the two cDNA sequences reveals two nucleotide differences, predicting one amino acid difference, in the region encoding amino acids 124–480 of the mature bovine β-subunit.

ACKNOWLEDGMENTS

We thank Dr. B. Roe, University of Oklahoma, for synthesizing the oligonucleotide probe. We thank Dr. G. S. McKnight, University of Washington, for the generous gift of the bovine heart cDNA library and Dr. M. Douglas, The University of Texas Health Science Center at Dallas, for the generous gift of the cloned *atp2* gene of *S. cerevisiae*. We thank M. Wing and Y.-C. Xu for help with screening the bovine cDNA library and C. Bailey for excellent editorial assistance.

REFERENCES

Amzel, L. M., & Pedersen, P. L. (1983) *Annu. Rev. Biochem. 52*, 801–824.

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., & Young, I. G. (1981) *Nature (London) 290*, 457–465.

Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W., & Clayton, D. A. (1981) *Cell (Cambridge, Mass.) 26*, 167–180.

Boutry, M., & Chua, N.-H. (1985) *EMBO J. 4*, 2159–2165.

Chen, E. Y., & Seeburg, P. H. (1985) *DNA 4*, 165–170.

Chirgwin, J. M., Przybyla, A. F., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Douglas, M., McCammon, M., & Vassarotti, A. (1986) *Microbiol. Rev. 50*, 166–178.

Fearnley, I. M., & Walker, J. E. (1986) *EMBO J. 5*, 2003–2008.

Feinberg, A. P., & Vogelstein, B. (1983) *Anal. Biochem. 132*, 6–13.

Futai, M., & Kanazawa, H. (1983) *Microbiol. Rev. 47*, 285–312.

Gait, M. J., Mathes, H. W. D., Singh, M., Sproat, B. S., & Titmas, R. C. (1982) *Nucleic Acids Res. 10*, 6243–6254.

Gay, N. J., & Walker, J. E. (1985) *EMBO J. 4*, 3519–3524.

Guerry, P., LeBlanc, D. J., & Falkow, S. (1973) *J. Bacteriol. 116*, 1064–1066.

Hanahan, D., & Meselsen, M. (1980) *Gene 10*, 63–71.

Karlin-Neumann, G., & Tobin, E. M. (1986) *EMBO J. 5*, 9–13.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Kobayashi, K., Iwasaki, Y., Sasaki, T., Nakamura, K., & Asahi, T. (1986) *FEBS Lett. 203*, 144–148.

Kozak, M. (1984) *Nucleic Acids Res. 12*, 857–872.

Lutcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., & Scheele, G. A. (1987) *EMBO J. 6*, 43–48.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T., Inayama, S., & Omura, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4647–4651.

Ohta, S., & Kagawa, Y. (1986) *J. Biochem. (Tokyo) 99*, 135–141.

Proudfoot, N. J., & Brownlee, G. (1976) *Nature (London) 263*, 211–214.

Rigby, P., Dieckman, N., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.

Roise, D., Horvath, S. J., Richards, J. H., Tomich, J. M., & Schatz, G. (1986) *EMBO J. 5*, 1327–1334.

Runswick, M. J., & Walker, J. E. (1983) *J. Biol. Chem. 258*, 3081–3089.

Runswick, M. J., Powell, S. J., Nyren, P., & Walker, J. E. (1987) *EMBO J. 6*, 1367–1373.

Sadler, I., Suda, K., Schatz, G., Kaudewitz, F., & Haid, A. (1984) *EMBO J. 3*, 2137–2143.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Schatz, G. (1987) *Eur. J. Biochem. 165*, 1–6.

Schmidt, G. W., & Mishkind, M. L. (1986) *Annu. Rev. Biochem. 55*, 879–912.

Senior, A. E., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 105–124.

Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.

Suggs, S. V., Wallace, R. B., Hirose, T., Kawashima, K., & Itakura, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6613–6617.

Sundaralingham, M., Drendel, W., & Greaser, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7944–7947.

Takeda, M., Vassarotti, A., & Douglas, M. G. (1985) *J. Biol. Chem. 260*, 15458–15465.

Terwilliger, T. C., & Eisenberg, D. (1982) *J. Biol. Chem. 257*, 6016–6022.

Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201–5205.

Tzagoloff, A., & Myers, A. M. (1986) *Annu. Rev. Biochem. 55*, 249–285.

Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G., & McKnight, G. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1300–1304.

Vassarotti, A., Chen, W.-J., Smagula, C., & Douglas, M. G. (1987) *J. Biol. Chem. 262*, 411–418.

von Heijne, G. (1986) *EMBO J. 5*, 1335–1342.
Walker, J. E., Saraste, M., & Gay, N. J. (1984) *Biochim. Biophys. Acta 768*, 164–200.
Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., & Tybulewicz, V. L. J. (1985) *J. Mol. Biol. 184*, 677–701.
Wallace, D. C., Ye, J., Neckelmann, S. N., Singh, G., Webster, K. A., & Goldberg, B. D. (1987) *Curr. Genet. 12*, 81–90.
Ward, M., & Turner, G. (1986) *Mol. Gen. Genet. 205*, 331–338.

Structure of the Chromosomal Copy of Yeast ARS1†

D. Lohr*,‡ and T. Torchia§,‖

Department of Chemistry, Arizona State University, Tempe, Arizona 85287-1604, and Department of Biological Chemistry, Milton Hershey Medical Center, Penn State University, Hershey, Pennsylvania 17033

Received September 28, 1987; Revised Manuscript Received January 20, 1988

ABSTRACT: We have used deoxyribonuclease I (DNase I) and methidium-propyl-EDTA·Fe(II) digestion to characterize the chromosomal structure of the single-copy autonomously replicating sequence ARS1. The major feature of this chromatin is a region of strong hypersensitivity to both cleavage agents. The hypersensitive region contains most of the DNA sequences which have been suggested by in vitro mutagenesis studies [Celniker, S., Sweder, K., Srienc, F., Bailey, J., & Campbell, J. (1984) *Mol. Cell. Biol. 4*, 2455–2466] to be important in ARS function. It lies at the downstream end of the *TRP1* gene. A chromosomal DNase I footprinting analysis was carried out on the hypersensitive region. These data give direct evidence for several localized DNA/protein contacts within the hypersensitive region. The most prominent of these chromatin-dependent contacts is located on the functionally most important 11 base pairs of ARS DNA. On the *TRP1* side of the hypersensitive region, there are positioned nucleosomes. On the other side of the hypersensitive region, there is a complex (and possibly heterogeneous) structure.

Functionally important regions like centromeres, telomeres, replication origins, and gene regulatory sequences appear to be marked in the chromosome by anomalous chromatin structures. For example, centromeres and telomeres show regions of strong nuclease protection surrounded by precisely positioned nucleosomes (Bloom & Carbon, 1982; Palen & Cech, 1984). Gene regulatory sequences (Elgin, 1982) and replication origins are regions of nuclease hypersensitivity (Varshavsky et al., 1978; Scott & Wigmore, 1978; Borchsenius et al., 1981; Palen & Cech, 1984). The hypersensitive regions around replication origins have received less attention than the hypersensitive regions associated with gene regulatory sequences.

DNA sequences which are able to confer autonomous replication on a plasmid have been identified in yeast [cf. Stinchcomb et al. (1979)]. For this and other reasons (Williamson, 1985; Brewer & Fangman, 1987), these autonomously replicating sequence (ARS)[1] elements are strong candidates for origins of yeast DNA replication.

Chromatin structures of two ARS elements, 2 μm (Livingston, 1982; Veit & Fangman, 1985) and ARS1 (Thoma et al., 1984; Long et al., 1985), have been analyzed. ARS1 is the more thoroughly studied of the two. Using micrococcal nuclease and DNase I, Thoma et al. (1984) concluded that the ARS1 region contains precisely positioned nucleosomes lying on both sides of a central nuclease-hypersensitive region. Long et al. (1985), using only MNase digestion, confirmed nucleosome positioning in one of the regions described by Thoma et al. (1984) but concluded that the other region is nonnucleosomal.

In both of the previous analyses, ARS1 was present on an extrachromosomal plasmid, the *TRP1*–ARS1 circle. Since ARS1 is normally located on the chromosome, it is of interest to determine the structure of the chromosomal copy and to compare it to the structure of the plasmid-associated ARS. This comparison is of general interest, for studies of other eukaryotic replication origins have also involved nonchromosomal origins, e.g., SV40 or polyoma viral origins (Varshavsky et al., 1978; Scott & Wigmore, 1978), or the extrachromosomal rDNA origins of *Tetrahymena* (Borchsenius et al., 1981; Palen & Cech, 1984). This paper describes such a chromosomal analysis of yeast ARS1.

In addition to the differences in subject (chromosomal copy instead of a plasmid-associated ARS), the methodologies used in this study differ in several ways from those used in previous analyses of ARS1. First, we analyze the ARS when present in single copy. This avoids the possibility of structural heterogeneity arising from the presence of multiple copies. In previous analyses of ARS chromatin structure, the ARS sequences were present in multiple copies. Second, this analysis is carried out with yeast nuclei. Previous analyses of ARS1 were carried out on whole cell lysates. Lastly, we use DNase I and the sequence neutral cleavage agent MPE·Fe(II) (Hertzberg & Dervan, 1984) to lessen the problems associated with sequence-specific digestion.

† Supported by U.S. Public Health Service Research Career Development Award Ca00911 and Research Grant GM37788 to D.L. and by Research Grant GM27925 to T.T.

‡ Arizona State University.
§ Penn State University.
‖ Present address: Department of Neurology, School of Medicine, University of California, San Francisco, CA 94143-0518.

[1] Abbreviations: bp, base pair(s); DNase I, deoxyribonuclease I (EC 3.1.4.5); MPE·Fe(II), methidium-propyl-EDTA·Fe(II); MNase, micrococcal nuclease (EC 3.1.4.7); ARS1, autonomously replicating sequence 1; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/88/0427-3961$01.50/0