# Two Bovine Genes for Mitochondrial ADP/ATP Translocase Expressed Differently in Various Tissues†

Steven J. Powell,‡ Simon M. Medd,§ Michael J. Runswick, and John E. Walker*
*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.*
*Received June 20, 1988; Revised Manuscript Received August 19, 1988*

ABSTRACT: Two different bovine cDNAs have been characterized that encode closely related homologues of the mitochondrial membrane carrier protein ADP/ATP translocase. One of them codes for the protein that has been characterized previously from bovine heart mitochondria, and the other codes for a protein that differs from it in 33 amino acids out of 297. Including the base substitutions required to bring about these changes in amino acid sequence, the coding regions of the cDNAs differ at 184 positions. In addition, they are extensively diverged in their 3′ noncoding sequences, which differ greatly in both length and sequence, and these segments of the cDNAs have been used as hybridization probes to demonstrate that the expression of the two genes giving rise to the two proteins is very different in various bovine tissues. Expression of one gene predominates in heart muscle and that of the other in intestine. Hybridization experiments with digests of genomic DNA have shown the presence of numerous sequences related to the two cDNAs in both the bovine and human genomes. Some of these probably arise from pseudogenes, but three expressed genes have been detected in the human genome. The study of the regulation of the expression of these genes may help to illuminate the basis of tissue-specific human mitochondrial diseases which arise because of defects in mitochondrial enzymes only in the affected tissue and not in other tissues of the same individual.

Mitochondrial electron-transport complexes and ATP synthase are in the inner membrane of the organelle. In mammals, they are comprised of more than 50 different proteins of which 13 are encoded in mitochondrial DNA (Anderson et al., 1981, 1982; Bibb et al., 1981; Chomyn et al., 1985, 1986; Fearnley & Walker, 1986, 1987). The remainder are products of nuclear genes and are imported into the organelle (Schatz & Butow, 1983). Immunological studies of the electron-transfer complex cytochrome *c* oxidase showed the presence in various rat tissues of different isoforms of its nuclear-encoded subunits (Kuhn-Nentwig & Kadenbach, 1985), presumably the products of multigene families that are subject to tissue-specific regulation of expression. Other evidence for nuclear gene families coding for mitochondrial proteins came from studies of the ATP synthase complex. It was found that in both cows and humans the dicyclohexylcarbodiimide-reactive proteolipid, a membrane protein subunit of the $F_o$ sector of the enzyme, has at least two expressed genes called P1 and P2 (Gay & Walker, 1985; Farrell & Nagley, 1987; Dyer & Walker, 1988) and numerous spliced pseudogenes (Dyer & Walker, 1988; Dyer et al., 1988), and hybridization experiments indicated the presence in the human and bovine genomes of multiple genes for other components of ATP synthase (Walker et al., 1987a). Of particular interest was the finding that the P1 and P2 genes are expressed at different relative levels in various bovine tissues (Gay & Walker, 1985) since it suggests a possible explanation of human tissue-specific mitochondrial myopathies and neuropathies. Biochemical analyses of tissues from patients with these conditions have shown that often the malady is associated with a defective electron-transport complex in the diseased tissue, whereas in other tissues in the same individual the mitochondria function normally (Morgan-Hughes, 1986; Wallace, 1986; Clark et al., 1987; Capaldi, 1988).

The present paper describes the presence in the bovine genome of multiple nuclear genes for the ADP/ATP translocase, a major component of the inner mitochondrial membrane. This is a transport protein of 297 amino acids which takes ATP from the mitochondrial matrix across the inner membrane to the intermembrane space and carries ADP back (Klingenberg, 1985a,b). As described below, two cDNAs corresponding to different genes, named T1 and T2, have been characterized from a bovine cDNA library. The genes are expressed in widely different ratios in bovine tissues. Transcripts of the T1 gene are found predominantly in heart whereas T2 is overwhelmingly expressed in intestine. As described elsewhere, the two bovine cDNAs have been used to isolate the corresponding human genes, which have been fully sequenced (Cozens et al., 1988), and also to isolate human homologues of the bovine cDNAs from a liver library (Houldsworth & Attardi, 1988). In all, three expressed genes for ADP/ATP translocase have been detected in humans (Battini et al., 1987; Neckleman et al., 1987; Houldsworth & Attardi, 1988; Cozens et al., 1988), and as the bovine homologues, their expression also is regulated differently in various tissues.

## Materials and Methods

*Oligonucleotide Synthesis.* A mixture of 24 oligonucleotides 17 bases long with the sequence 3′ACCTACTA$^{R}$/$_{T}$CGNGTYTG5′ was synthesized by a solid-phase phosphotriester method (Gait et al., 1982; Sproat & Bannwarth, 1983). This corresponds to the protein sequence Trp-Met-Ile-Ala-Gln-Thr, residues 213–218 in the translocase (Aquila et al., 1982). It is estimated to have a minimum melting temperature of 48 °C assuming that each G·C and A·T base pair contributes 4 and 2 °C, respectively (Suggs et al., 1981). Unique synthetic oligonucleotides 17 bases in length used as sequencing primers were synthesized by T. V. Smith with the aid of an Applied Biosystems 320B oligonucleotide synthesizer.

*cDNA Libraries.* Two different libraries were investigated. A plasmid library in pUC8 was prepared from mRNA obtained from bovine heart and liver (Gay & Walker, 1985).

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02845.

* To whom correspondence should be addressed.
‡ Present address: ICI Pharmaceuticals Division, Northwich, Cheshire, U.K.

§ Supported by an MRC research studentship.

0006-2960/89/0428-0866$01.50/0 

A bovine brain library cloned in λgt10 was obtained from Dr. A. P. Jackson (Jackson et al., 1987).

*Screening cDNA Libraries.* Recombinants in the plasmid library were grown up and transferred to Pall Biodyne A nylon membranes. The oligonucleotide mixture (50 pmol) was radiolabeled using polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (specific activity approximately 3000 Ci/mmol) and purified by electrophoresis through a 20% polyacrylamide gel containing 6 M urea and 90 mM Tris (pH 8.3), 90 mM boric acid, and 25 mM EDTA. It was eluted and purified further by centrifugation through a column of Sephadex G50 (1 mL). Experimental conditions for treatment of the membranes and for hybridization with the probe have been described earlier (Gay & Walker, 1985) except that the hybridizations were performed at 43 °C (5 °C below the minimum melting temperature). Usually 10–20 filters were probed at the same time in about 100 mL of the hybridization buffer containing all of the recovered radiolabeled probe. From this experiment, isolates pBHL-T1.1, pBHL-T1.2, and pBHL-T2.1 were characterized. Only the first of these was found to encode a complete ADP/ATP translocase (see Figure 1). In order to try to obtain a full-length isolate of the T2 isoform, the bovine heart and liver cDNA library was screened again, this time using as a hybridization probe the insert from the partial clone, pBHL-T2.1, but to no avail. Therefore, it was decided to investigate a different cDNA library derived from bovine brain mRNA. Recombinants in this library were grown on *Escherichia coli* C600Hfl in petri dishes (13.5 cm diameter) for 5–6 h at 37 °C. Four nitrocellulose filters were placed sequentially on each plate, and they were left in contact with the plate for 1, 2, 4, and 6 min, respectively. Then they were treated according to Benton and Davis (1977). One pair of duplicate filters from each set was screened with a "prime-cut" probe derived from the 5′-terminal region of the partial clone isolated in pBHL-T2.1 (bases 704 to the *Sac*I site at base 941; see Figure 2). "Prime-cut" probes were synthesized from single-stranded DNA cloned in M13 vectors according to Farrell et al. (1983). About 1–2 μg of DNA and radioactive [$\alpha$-$^{32}$P]dATP or dCTP (800 Ci/mmol) were used in each synthesis. The second pair from each set was screened with a probe from the 3′ end of pBHL-T2.1 (bases 942–1367). Filters were prehybridized for 2 h at 65 °C with a solution containing 5 × Denhardt's solution [which contains 1 mg/mL each of poly(vinylpyrrolidone), bovine serum albumin (fraction V), and ficoll], 6 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.5% *N*-laurylsarcosine, and yeast RNA (200 mg/mL). Hybridization was performed at 65 °C for 18 h in the same solution supplemented with 10% dextran sulfate and containing the radioactive probe. Filters were autoradiographed as above. Coincident hybridizing plaques that were detected on all four filters in each set were rescreened under the same conditions, and positively hybridizing recombinants were plaque-purified. DNA was prepared from recombinants by the plate lysate method (Maniatis et al., 1982). About 0.5 × $10^6$ recombinants distributed on 10 plates were screened by this procedure. More than 20 positively hybridizing recombinants were identified, of which 3 were investigated in detail (see Figure 1).

*Subcloning and DNA Sequencing.* DNA sequence analysis was carried out by the method of Sanger et al. (1977) as modified by Biggin et al. (1983). Inserts from pUC plasmids and from λgt10 recombinants were excised with *Eco*RI and *Bam*HI together and *Eco*RI alone, respectively. The resulting fragments were cloned into appropriate M13mp8, M13mp9, and pUC vectors. Then the sequences at the ends of fragments were determined from the M13 recombinants; the pUC recombinants were used to amplify the fragments. The sequences of T1 recombinants, which proved to be essentially full-length, were completed processively by the use of 11 synthetic sequencing primers all 17 bases in length. The original isolate of T2, pBHL-T2.1, contained a partial clone for a translocase, and the sequence was determined in the flanking regions of the insert. It was used to isolate related but longer recombinants from the λgt10 library as described above. The sequence of T2 was determined by making a random library from sonicated fragments of λBB-T2.1 (see Figure 1; Deininger, 1983), and the sequence was established by using the random strategy (Bankier & Barrell, 1983). A further five synthetic primers also were needed to complete the sequence. Problematic regions in the sequences of both T1 and T2 were determined by the use of either deoxyinosine triphosphate (Mills & Kramer, 1979) or deoxy-7-deazaguanosine triphosphate (Mizusawa et al., 1986) in the place of dGTP in the sequencing reaction mixtures.

*Hybridizations with RNA.* Total heart mRNA was extracted from various fresh bovine tissues according to Chirgwin et al. (1979). As soon as possible after slaughter of the animal, small pieces of tissue (heart, liver, lung, brain, intestine, and kidney) were frozen in liquid $N_2$ and transported to the laboratory. Then they were pulverized under liquid $N_2$ and homogenized for 60 s with a Polytron homogenizer in 4 M guanidinium thiocyanate containing 25 mM sodium citrate, pH 7.0, and 0.1 M EDTA. Poly(A+) RNA was prepared from total RNA by the method of Aviv and Leder (1972) except that NaCl was replaced by LiCl (Maniatis et al., 1982). Hybridizations with RNA were performed as follows. RNA samples were treated with glyoxal and fractionated on a 1.4% HGT–agarose gel submerged in a stirred solution of 10 mM sodium phosphate, pH 7.0. RNA was transferred to Hybond N (Amersham International PLC, U.K.) and cross-linked to the membrane by UV irradiation. Hybridization was performed with "prime-cut" probes (Farrell et al., 1983). For a description of the probes used in these experiments, see the legend to Figure 4. Membranes were washed 4 times at 65 °C in 6 × SSC, twice in 2 × SSC, and twice in 0.2 × SSC. Autoradiography was carried out for 1–3 days at −70 °C with preflashed film and an intensifying screen.

*Hybridizations with Genomic DNA.* The preparation of human and bovine genomic DNA has been described elsewhere (Walker et al., 1987a). Samples of human and bovine DNA (20 μg/slot) were digested at 37 °C for 2.5–3.0 h in 400 μL of buffer with restriction endonucleases, including *Bam*HI, *Eco*RI, and *Pst*I. The DNA was precipitated at −20 °C with ethanol and sodium acetate (final concentration 0.3 M). Electrophoresis of digests and transfer of separated fragments to nitrocellulose membranes were performed according to Southern (1975) as described in detail elsewhere (Walker et al., 1987a). Prehybridization of membranes was carried out for 2–3 h at 56 °C by agitating the membranes in a solution containing 6 × SSC, 5 × Denhardt's solution, sonicated salmon sperm DNA (100 μg/mL), and 0.5% sarkosyl. Hybridization was performed for 18 h at 65 °C in the presence of the same solution containing 10% dextran sulfate and the radiolabeled probe. Then filters were washed twice at 65 °C in 2 × SSC and twice in 0.2 × SSC, both solutions containing 0.5% sarkosyl. Filters were autoradiographed at −70 °C for 24–96 h with preflashed film (Fuji RX 150).

## Results and Discussion

*Cloning and DNA Sequence Analysis.* In the first experiments to isolate a cDNA clone for ADP/ATP translocase,

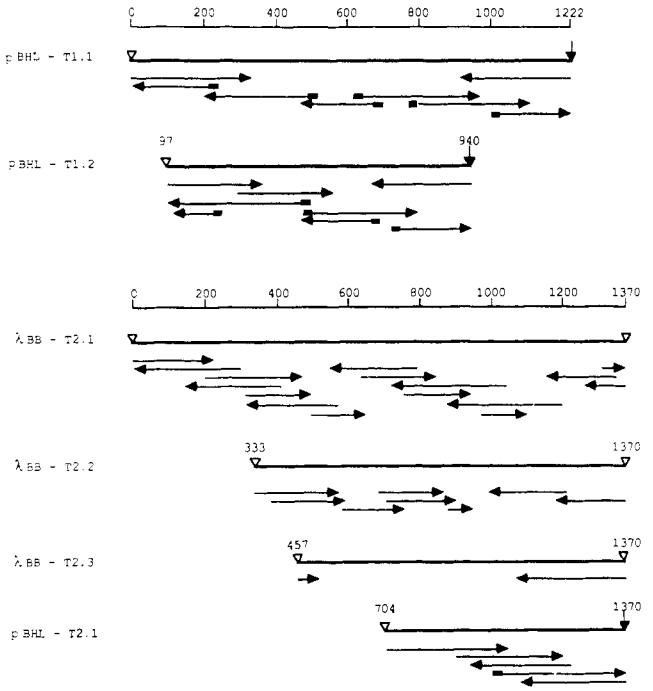


FIGURE 1: Extent of inserts in cDNAs encoding bovine ADP/ATP translocases and of sequences determined in subclones derived from them. The scales are in bases. The clones were derived from bovine cDNA libraries in either plasmid pUC8 or λgt10 made with mRNA derived from heart and liver together (BHL) and brain (BB), respectively. The upper part of the diagram summarizes the sequence analysis of two clones, pBHL-T.1 and pBHL-T1.2, isolated from the bovine heart and liver library. The inserts are flanked by an *Eco*RI (▽) and a *Bam*HI site (↓) in the polylinker of the vector, and they were excised by digestion with these two enzymes. The sequences were generated by the use of unique synthetic oligonucleotide primers, each 17 bases long, indicated by black boxes. Isolate pBHL-T1.2 is a partial clone extending from bases 97 to 940. Its 3′ end, however, is polyadenylated after base 940. In the lower part of the diagram, the sequence analysis of clones encoding the T2 isoform is summarized. Isolates λBB-T2.1 to λBB-T2.3 and pBHL-T2.1 were isolated from the bovine brain and heart with liver libraries, respectively. Flanking sequences were determined in all isolates. The sequences of λBB-T2.1 and λBBT2.2 were completed by the sequence analysis of fragments generated by sonication of the inserted DNAs. A synthetic primer, indicated by a black box, was employed in the sequencing of pBHL-T2.1. All of the subclones that were sequenced are not shown in this figure, which, however, contains sufficient clones to establish the sequence minimally in both senses of the DNA.

a bovine cDNA library derived from heart and liver RNA was investigated. The library had been cloned into a plasmid vector (Gay & Walker, 1985) and was screened by hybridization with the mixture of 24 synthetic oligonucleotides designed on the basis of the known protein sequence of the translocase (Aquila et al., 1982; see Materials and Methods). Three hybridizing recombinants, named pBHL-T1.1, pBHL-T1.2, and pBHL-T2.1, were detected (Figure 1), and DNA sequence analysis (Figure 2) revealed that the inserts in the first two were related to each other but not to that in pBHL-T2.1. Its insert was derived from a different RNA species. The corresponding genes have been named T1 and T2. The insert in pBHL-T1.1 codes for a protein sequence identical with that of the ADP/ATP translocase described by Aquila et al. (1982), whereas the insert in pBHL-T1.2 encodes a sequence related to, but not identical with, the C-terminal 84 amino acids of the same protein. This partial DNA sequence is nucleotides 704–1367 of the complete T2 cDNA presented in Figure 2. These experiments have been reported briefly before (Walker et al., 1987b). In order to try to isolate a full-length cDNA for T2, the plasmid library was rescreened with a probe extending from the 5′ end of the insert in pBHL-T2.1 to the *Sac*I site after nucleotide 942, but with no success. So a second library derived from bovine brain mRNA was screened with the same probe. Thereby, 20 positively hybridizing recombinants were identified, and 3 of them, λBB-T2.1–λBB-T2.3 (see Figure 1), were characterized in detail. Recombinant λBB-T2.1 proved to contain the complete coding sequence for the T2 translocase, the whole of the 3′ noncoding region derived from the mRNA, and a fairly extensive sequence from the 5′ noncoding region. The sequences of the T1 and T2 cDNAs presented in Figure 2 were fully determined in both senses of the DNA, and on average, each base in T1 and T2 was determined 6 and 12 times, respectively. Through the region of overlap of the clones of T2 from heart/liver and brain, the sequences are exactly in agreement.

The nucleotide sequences in the coding regions of the cDNAs for T1 and T2 are strongly conserved, and they differ at 184 nucleotides out of 894. More than half of these changes are found in silent positions of codons, and most of the remainder give rise to conservative amino acid substitutions, the protein sequences of the T1 and T2 translocases differing in 33 amino acids out of 298 (including the N-terminal methionine residue). A model of the ADP/ATP translocase has been proposed in which the protein has six transmembrane helical segments linked by extramembranous loops (Saraste & Walker, 1982). It is notable that most of the differences in sequence between T1 and T2 are found in these proposed loops (see Figure 3).

In contrast to the coding regions, the 3′ noncoding regions of the T1 and T2 cDNAs are at best weakly related in sequence and also differ in length, being 286 and 389 nucleotides, respectively. Thus, these regions provide specific probes for genes and transcripts of T1 and T2. Both contain the sequence AATAAA 13 and 12 bases, respectively, before the poly(A) tails, presumed to be signals for polyadenylation (Proudfoot & Brownlee, 1976). Poly(A) addition in T1 also occurs 17 bases after the sequence ATTAAA (bases 913–918). This was demonstrated previously by the characterization of a partial cDNA clone for bovine ADP/ATP translocase (Rasmussen & Wohlrab, 1986) which corresponds to bases 633–934 in the T1 cDNA sequence presented in Figure 2, although its sequence differs in seven silent positions of codons (see the legend to Figure 2). It is identical in the 3′ noncoding region from bases 909–934 to the T1 clone described in the present work.

The 5′ noncoding region present in the T1 cDNA is only 11 bases in length, and that determined in the equivalent region of the T2 cDNA described in Figure 2 is 63 bases long, and they both may be incomplete. Therefore, the relationship between the 5′ regions of the bovine T1 and T2 mRNAs cannot be assessed.

*Mitochondrial Import of ADP/ATP Translocase.* The mature T1 ADP/ATP translocase has two posttranslational modifications: the N-terminal serine residue is $N^{\alpha}$-acetylated (Aquila et al., 1982), and residue 51 is $N^{\epsilon}$-trimethyllysine (Klingenberg, 1985a). In the protein sequences encoded in the cDNAs, this serine is preceded by a methionine residue, and the question arises of whether it is the translational initiator. Most nuclear-encoded mitochondrial proteins are translated as precursors with N-terminal extensions which direct the protein into the mitochondrion. The extension is removed by proteolysis during the import process (Schatz & Butow, 1983). However, fungal ADP/ATP translocases are homologous to the mammalian proteins and are exceptional in that they are imported into mitochondria and yet they do not have a processed import sequence at their N-termini; only

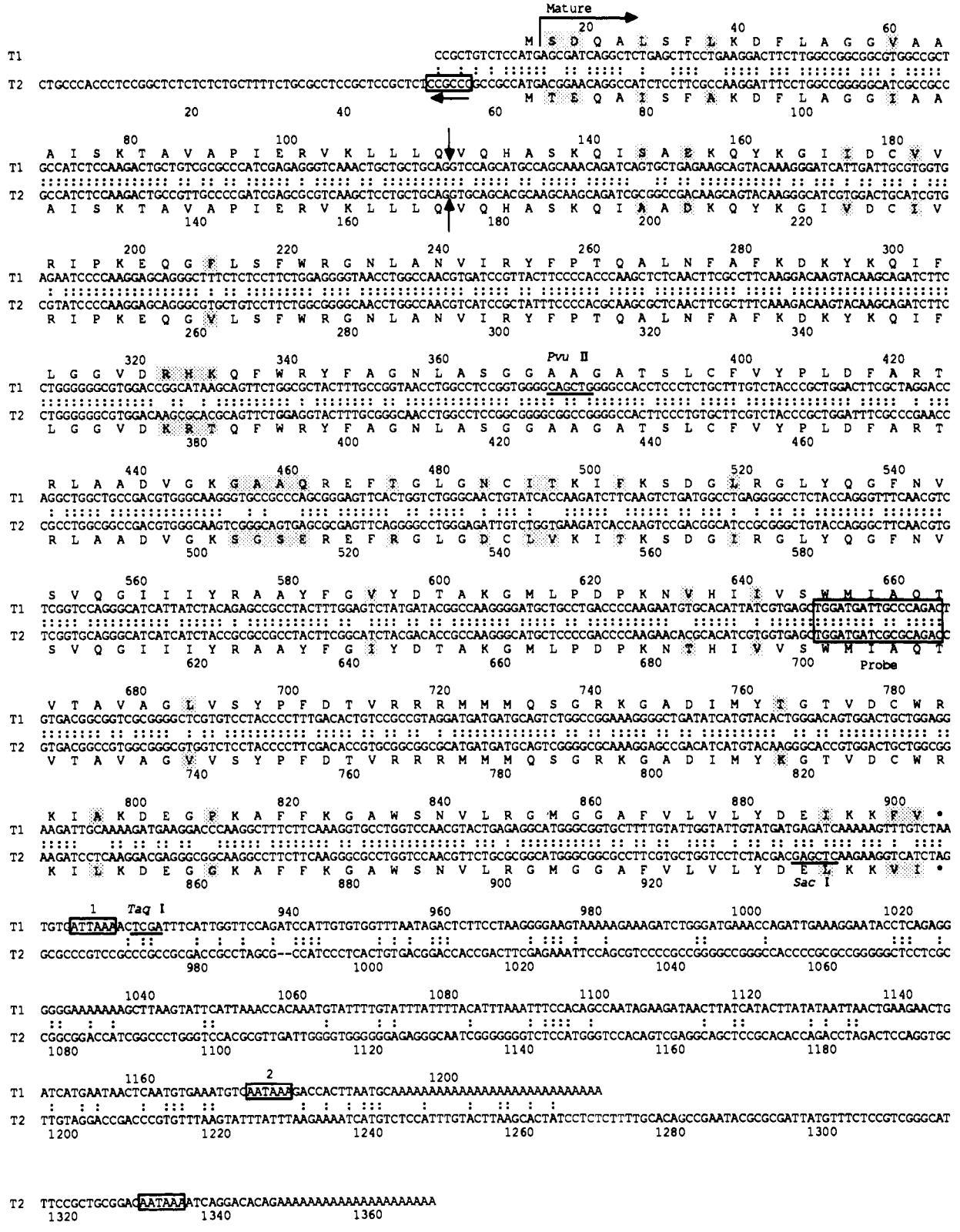


FIGURE 2: DNA sequences of bovine cDNA and derived protein sequences of T1 and T2 translocases. The nucleotide sequences were aligned with the computer program NUCALN (Wilbur & Lipman, 1983). The numbers refer to the nucleotide sequence, and colons denote identities. Dashes in the nucleotide sequence indicate insertions which have been introduced to improve alignment of the 3′ noncoding regions. Differences in amino acid sequence between the two proteins are shaded. The N-terminus of the mature T1 protein is shown (Aquila et al., 1982). The boxed hexanucleotide sequence near the 5′ end of T2 is the complement of a sequence associated with binding sites for the transcription factor SP1 (Kadonaga et al., 1986). The vertical arrows indicate the positions of introns I in the homologous human genes (Cozens et al., 1988). The boxed sequences in the 3′ regions are likely to be signals for polyadenylation (Proudfoot & Brownlee, 1976). Site 1 in T1 (nucleotides 913–918) is an alternative polyadenylation site observed in clone pBHL-T1.2, and earlier by Rasmussen and Wohlrab (1986). Site 2 was observed in clone pBHL-T1.1. The DNA sequences in the coding regions of T1 and T2 that hybridized with the mixed oligonucleotide probe are boxed. Also shown are *Pvu*II and *Taq*I sites in T1 and a *Sac*I site in T2 that were important in the preparation of "prime-cut" probes used in RNA and DNA hybridization experiments (see Figures 4 and 5). A partial cDNA sequence for T1 described by Rasmussen and Wohlrab (1986) extends from nucleotide 633 to nucleotide 934 and is followed by the sequence $A_{16}$. It was determined in one sense of the DNA only and differs at seven positions from the sequence reported in this figure. At nucleotides 647, 674, 677, 680, 683, 734, and 743, Rasmussen and Wohlrab (1986) report A, C, T, T, A, A, and T, respectively.

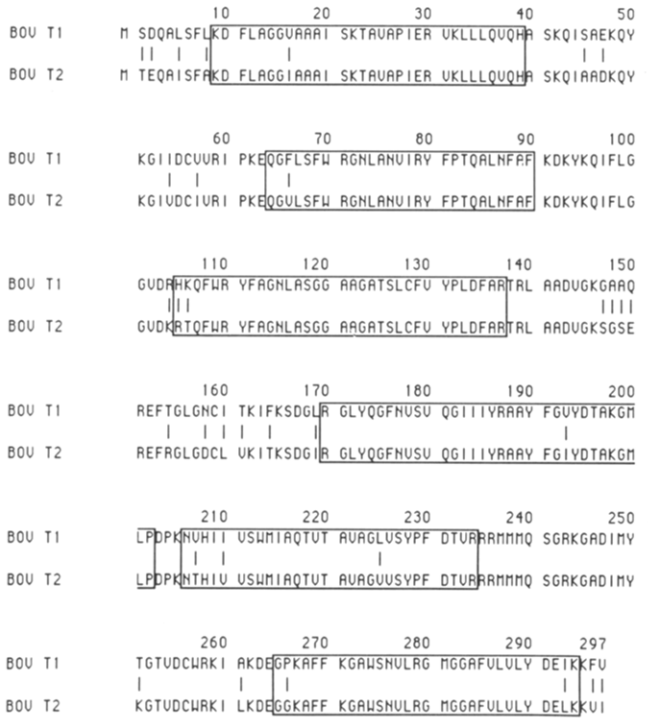


FIGURE 3: Differences between the protein sequences of bovine ADP/ATP translocases T1 and T2. The computer program PRTALN (Wilbur & Lipman, 1983) was used to align them. Differences between the two sequences are shown by vertical lines. The proteins are presented with their initiator methionine residues. This is known to be removed from T1, and the N-terminal serine residue is N-acetylated, but there is no evidence about the N-terminus of the T2 protein. In the T1 protein, residue 51 is $N^{\epsilon}$-trimethyllysine. The six boxed regions represent transmembrane $\alpha$-helical segments (Saraste & Walker, 1982).

their initiator methionine residues are removed posttranslationally (Arends & Sebald, 1984; Adrian et al., 1986). So it seems likely that these methionine residues in bovine T1 and T2 are also the translational initiation codons. This conclusion has gained further support from the sequences of the human T1 and T2 genes (Cozens et al., 1988) and the T1 cDNA (Neckelman et al., 1987). These show that the proposed initiator methionine codons, which are conserved in the human homologues, are not preceded by DNA that could code for a segment of protein with features resembling import sequences which, although highly variable in length and sequence, always appear to have a net positive charge and rarely contain acidic residues.

*Expression of Bovine T1 and T2 Translocase Genes in Tissues.* Earlier investigations of the expression of the bovine P1 and P2 genes coding for different precursors of the proteolipid subunit of mitochondrial ATP synthase showed that their transcripts were present in different ratios in various bovine tissues (Gay & Walker, 1985). A similar investigation was performed on the expression of the T1 and T2 translocase genes. We examined the representation of their transcripts in total RNA and in poly(A+) RNA prepared from bovine intestine, kidney, heart, liver, lung, and brain. Hybridization of the fractionated samples using T1- and T2-specific probes derived from the highly diverged 3′ noncoding regions of the cDNAs revealed strikingly different distributions of their mRNAs (see Figure 4). T1 mRNA is present predominantly in heart, and to a lesser extent in kidney, whereas T2 mRNA was found in intestine and kidney, and at lower levels in other tissues also. These experiments clearly demonstrate differences in expression of T1 and T2 in heart, intestine, and kidney, but the absence of strong signals with either probe in RNA

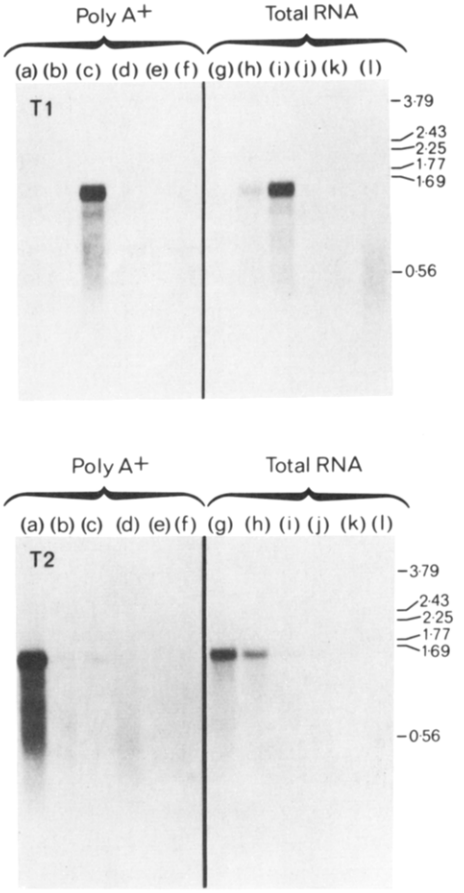


FIGURE 4: Hybridization of specific probes for T1 and T2 translocases with RNA derived from various bovine tissues. Both of the probes employed were derived from the diverged sequences of the 3′ noncoding regions of the T1 and T2 cDNAs. The T1-specific probe extends from the *Taq*I site to the *Bam*HI site in the polylinker beyond the poly(A) tail (nucleotide 923 to polylinker). The T2-specific probe was a *Sac*I–*Bam*HI fragment extending from nucleotide 943 to the polylinker beyond the poly(A) sequence. In experiments with poly(A+) RNA, 2 $\mu$g of each mRNA was employed, and in the case of hybridizations with total RNA, 10 $\mu$g of RNA from each tissue was applied to the gel. Two separate gels were employed for separation of the RNAs shown in the two panels, poly(A+) and total RNA being run side by side on each gel as shown. The gels were run in parallel with aliquots of the same RNA preparations. RNA from the following tissues was used: (a and g) intestine; (b and h) kidney; (c and i) heart; (d and j) liver; (e and k) lung; (f and l) brain. The markers correspond to the positions of fragments of DNA from bacteriophage λ generated by digestion with the restriction enzyme *Nde*I.

preparations from other tissues is more difficult to evaluate. Lack of signal could be thought to arise for trivial reasons such as failure to load the sample on the gel or because of extensive degradation of RNA in the preparations. The first reason can be dismissed since the experiments were repeated, with the same outcome. Further experiments are required to investigate the second possibility, since there is evidence of degradation in some slots in Figure 4.

The sizes of both T1 and T2 mRNAs are estimated to be about 1.4 kb (using DNA markers) which indicates that the cDNA sequences are near to being full-length. A more weakly hybridizing band of about 1.2 kb was also detected in heart mRNA by hybridization with the T1-specific probe. The origin of this band is unclear at present, and it is possible that it arises from degradation of the 1.4-kb transcript. The presence in various bovine tissues of two species of RNA 1.4 and 1.2 kb in length which hybridize to a probe for ATP/ADP translocase which has now been shown to be part of T1 has been noted previously (Rasmussen & Wohlrab, 1986). The

levels and relative abundancies of these two mRNAs appear to differ between tissues, and this was taken as evidence for organ-specific forms of the mRNAs. From the two independent DNA sequences in the 3′ noncoding regions of T1, it now seems quite likely that these two RNA species are derived from transcripts of the same gene that have been polyadenylated at two different sites 260 bases apart (see previous section and Figure 2). In addition to the present work, evidence for the expression of different forms of ADP/ATP translocase has been provided earlier by immunological studies of the protein isolated from heart, liver, and kidney (Schultheiss & Klingenberg, 1984, 1985). Organ-specific determinants were detected, but with partial identity of the proteins from the various sources. Different levels of expression of human T1 in various tissues have also been noted (Neckelman et al., 1987).

A notable feature of the bovine T2 cDNA, but not of the bovine T1 cDNA, that could influence expression of the gene is the relatively high frequency of the dinucleotide CpG throughout its sequence. This is similar to both the human T1 and T2 genes which have a high concentration of the dinucleotide through regions of greater than 1 and 1.5 kb, respectively, that extend from beyond the 5′ noncoding regions present in the mRNA through the first exons and into the first introns, but not apparently further (Cozens et al., 1988). So in this respect the bovine cDNAs appear to differ from their human homologues, since it is absent from the bovine T2 cDNA and the CpG-rich region in T2 extends beyond the position of the first intron in the human gene (see Figure 1). We have suggested that these human sequences may be examples of "CpG" islands, which are associated with the 5′ ends of some housekeeping genes that are expressed in all tissues, and also with genes that are expressed only in specific cell types (Bird, 1986, 1987). It is thought that the cytosines in CpG islands are not methylated, whereas they are elsewhere, and that this influences their ability to bind various transcriptional factors. It is therefore of some interest to find that the 5′ noncoding sequence in the bovine T2 cDNA contains the hexanucleotide sequence CCGCCC (see Figure 2). This is a potential site for binding the transcriptional factor SP1, which can enhance transcription by RNA polymerase II by 10–20-fold (Kadonaga et al., 1986). Fifteen similar potential sites are associated with the 5′ region of the human T1 gene, and a further three potential sites with the T1 gene (Cozens et al., 1988). The evaluation of the significance of these sequences awaits further study.

*Number of Genes for ADP/ATP Translocase.* In order to investigate the number of different sequences coding for translocase-like proteins in the bovine and human genomes, digests of bovine and human DNAs were hybridized with probes derived from the 5′ and 3′ coding regions of T1 and T2, respectively. The sequences in these probes are conserved in the bovine T1 and T2 cDNAs, and both probes would be expected to detect T1- and T2-related sequences. In the experiments both with bovine DNA and with human DNA (see Figure 5), some bands of the same size were detected with both probes, indicating that at least part of the 5′ and the 3′ sequences related to the translocase are not further apart from the size of the fragment. In some of these cases, bands of apparently the same size in digests with the same enzyme hybridize with different intensities. Given that equal amounts of DNA were used in each digest, and that high molecular weight hybridizing bands that are diagnostic of partial digestion are not present, this probably arises because the 5′ end of the detected sequence is strongly related to the probe,

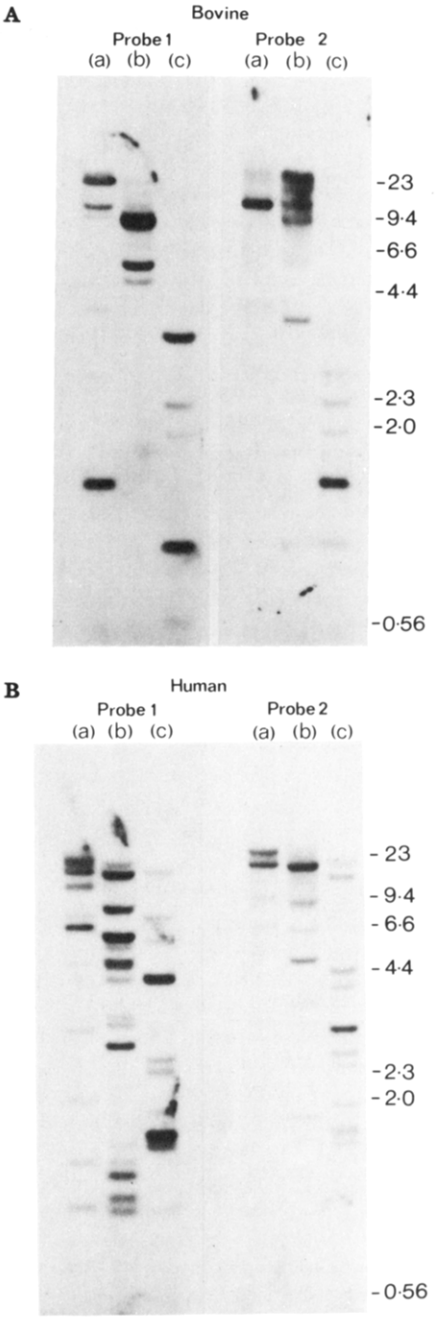


FIGURE 5: Hybridization of digests of bovine (A) and human (B) DNA with probes derived from bovine ADP/ATP translocase cDNAs T1 and T2. The following hybridization probes were employed. Probe 1 was taken from the coding region of the T1 cDNA and extends from the *Eco*RI site in the polylinker (to the 5′ side of nucleotide 1) to the *Pvu*II site at nucleotide 378. Probe 2 was taken from the coding region of the partial T2 cDNA clone pBHL-T2.1. It extends from the *Eco*RI site in the polylinker (to the 5′ side of the insert) to the *Sac*I site close to the 3′ end of the coding regions (bases 704–942). (See Figure 2 for positions of restriction enzyme sites in T1 and T2 cDNAs and Materials and Methods for other experimental details.) DNA was digested with the following restriction endonucleases: (a) *Bam*HI; (b) *Eco*RI; (c) *Pst*I. The markers are the positions of fragments of DNA from bacteriophage λ generated by digestion with the restriction enzyme *Hin*dIII. In panels A and B, two sets of equal samples taken from the same digests of bovine and human DNA, respectively, were separated in parallel on each gel, transferred to nitrocellulose membranes, and hybridized with the probes.

whereas the 3′ end is weakly related (or vice versa). In other cases where one band is present in one digest but not in the other, then presumably the 5′ and 3′ ends of the translocase-related sequence lie further apart than the size of the fragment, and this indicates an interrupted sequence. These

experiments indicate that the bovine genome contains between 3 and 7 sequences related to the probes, and the human genome even more, with 12–15 hybridizing bands evident in the various digests. Further hybridization experiments have been carried out to distinguish between T1- and T2-related human sequences (A. L. Cozens and J. E. Walker, unpublished results), and the genomic DNA sequences of human T1 and T2 are helpful in this respect also (Cozens et al., 1988). In the human genome, three expressed genes for ADP/ATP translocase have been detected. Two are counterparts of bovine T1 and T2, and the third, for which we propose the name T3, is expressed in increased amount in HL60 cells that have been growth-stimulated (Battini et al., 1987). Human T1 and T2 have been fully sequenced (Cozens et al., 1988), and these data provide a basis for further studies of their expression and may conceivably help in the understanding of the molecular basis of the tissue-specific myopathies and neuropathies.

These estimates of gene numbers do not distinguish between expressed genes and pseudogenes. Patterns of hybridization with digests of genomic DNA of comparable complexity have been noted earlier with other mammalian mitochondrial proteins including the bovine and human dicyclohexylcarbodiimide-reactive proteolipids (Dyer & Walker, 1988), the bovine oligomycin-sensitive conferral component (another component of ATP synthase; Walker et al., 1987a), and mouse cytochrome *c* (Limbach & Wu, 1985). In the case of the DCCD-reactive proteolipid, two different expressed genes have been detected in both the bovine (Gay & Walker, 1985) and human genomes (Farrell & Nagley, 1987; Dyer & Walker, 1988). In addition, in both species, at least 10 different spliced pseudogenes have been found (Dyer & Walker, 1988). The mouse has two functional cytochrome *c* genes, one expressed in all somatic tissues and the other only in testis, and in addition four pseudogenes have been characterized (Limbach & Wu, 1985). Not all mammalian mitochondrial proteins seem to have multiple expressed genes. For example, no evidence has been found for different isoforms of human and bovine cytochrome *c* oxidase subunit IV (Zeviani et al., 1987; Bachman et al., 1987), and preliminary hybridization studies are consistent with the presence of single genes for the ATPase inhibitor protein and factor 6, a component of ATP synthase (Walker et al., 1987a), although these experiments are not conclusive since distantly related sequences might not have been detected under the experimental conditions used.

## Acknowledgments

We thank T. V. Smith for synthesizing oligonucleotides and Dr. A. P. Jackson for a sample of the cDNA library derived from bovine brain.

**Registry No.** ADP/ATP translocase, 9068-80-8; DNA (ox ADP/ATP translocase T1 mRNA complementary), 117438-13-8; ADP/ATP translocase T1 (precursor), 117438-15-0; ADP/ATP translocase T1, 117438-16-1; DNA (ox ADP/ATP translocase T2 mRNA complementary), 117438-12-7; ADP/ATP translocase T2, 117438-14-9.

## References

Adrian, G. S., McCammon, M. T., Montgomery, D. L., & Douglas, M. G. (1986) *Mol. Cell. Biol. 6*, 626–634.

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

Anderson, S., de Bruijn, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., & Young, I. G. (1982) *J. Mol. Biol. 156*, 683–717.

Aquila, H., Misra, D., Eulitz, M., & Klingenberg, M. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 345–349.

Arends, H., & Sebald, W. (1984) *EMBO J. 3*, 377–382.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Bachman, N. J., Lomax, M. I., & Grossman, L. I. (1987) *Gene 55*, 219–229.

Bankier, A. T., & Barrell, B. G. (1983) in *Techniques in Nucleic Acid Biochemistry* (Flavell, R. A., Ed.) Vol. B508, pp 1–34, Elsevier, County Clare, Ireland, and New York.

Battini, R., Ferrari, S., Kaczmarek, L., Calabretta, B., Chen, S. T., & Baserga, R. (1987) *J. Biol. Chem. 262*, 4355–4359.

Benton, W. D., & Davis, R. W. (1977) *Science (Washington, D.C.) 196*, 180–182.

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

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Bird, A. P. (1986) *Nature (London) 321*, 209–213.

Bird, A. P. (1987) *Trends Genet. 3*, 342–346.

Capaldi, R. A. (1988) *Trends Biochem. Sci. (Pers. Ed.) 13*, 144–148.

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

Chomyn, A., Mariottini, P., Cleeter, M. W. J., Ragan, C. I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F., & Attardi, G. (1985) *Nature (London) 314*, 592–597.

Chomyn, A., Cleeter, M. W. J., Ragan, C. I., Riley, M., Doolittle, R. F., & Attardi, G. (1986) *Science (Washington, D.C.) 234*, 614–618.

Clark, J. B., Hayes, D. J., Byrne, E., & Morgan-Hughes, J. A. (1987) *Biochem. Soc. Trans. 15*, 626–627.

Cozens, A. L., Runswick, M. J., & Walker, J. E. (1988) *J. Mol. Biol.* (submitted for publication).

Deininger, P. L. (1983) *Anal. Biochem. 129*, 216–223.

Dyer, M. R., & Walker, J. E. (1988) *J. Mol. Biol.* (submitted for publication).

Dyer, M. R., Gay, N. J., & Walker, J. E. (1988) *J. Mol. Biol.* (submitted for publication).

Farrell, L. B., & Nagley, P. (1987) *Biochem. Biophys. Res. Commun. 144*, 1257–1264.

Farrell, P. J., Deininger, P. L., Bankier, A., & Barrell, B. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1565–1569.

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

Fearnley, I. M., & Walker, J. E. (1987) *Biochemistry 26*, 8247–8251.

Gait, M. J., Matthes, 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.

Houldsworth, J., & Attardi, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 377–381.

Jackson, A. P., Seow, H.-F., Holmes, N., Drickamer, K., & Parham, P. (1987) *Nature (London) 326*, 154–159.

Kadonaga, J. T., Jones, K. A., & Tijian, R. (1986) *Trends Biochem. Sci. (Pers. Ed.) 11*, 20–23.

Klingenberg, M. (1985a) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) Vol. 4, pp 511–553, Plenum, New York.

Klingenberg, M. (1985b) *Ann. N.Y. Acad. Sci. 456*, 279–288.

Kuhn-Nentwig, L., & Kadenbach, B. (1985) *Eur. J. Biochem. 149*, 147–158.

Limbach, K. J., & Wu, R. (1985) *Nucleic Acids Res. 13*, 617-620.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Mills, D. R., & Kramer, F. R. (1979) *Proc. Natl. Acad. Sci. U.S. A. 76*, 2232-2235.
Mizusawa, S., Nishimura, S., & Seela, F. (1986) *Nucleic Acids Res. 14*, 1319-1324.
Morgan-Hughes, J. A. (1986) *Trends Neurosci. (Pers. Ed.) 9*, 15-19.
Neckelmann, N., Li, K., Wade, R. P., Shuster, R., & Wallace, D. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7580-7584.
Proudfoot, N. J., & Brownlee, G. (1976) *Nature (London) 263*, 211-214.
Rasmussen, U. B., & Wohlrab, H. (1986) *Biochem. Biophys. Res. Commun. 138*, 850-857.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Saraste, M., & Walker, J. E. (1982) *FEBS Lett. 144*, 250-254.
Schatz, G., & Butow, R. A. (1983) *Cell (Cambridge, Mass.) 32*, 316-318.
Schultheiss, H. P., & Klingenberg, M. (1984) *Eur. J. Biochem. 143*, 599-605.
Schultheiss, H. P., & Klingenberg, M. (1985) *Arch. Biochem. Biophys. 239*, 273-279.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503-517.
Sproat, B. S., & Bannwarth, W. (1983) *Tetrahedron Lett. 24*, 5771-5774.
Suggs, S. V., Hirose, T., Miyake, T., Kawashima, E. H., Johnson, M. J., Itakura, K., & Wallace, R. B. (1981) in *Developmental Biology Using Purified Genes* (Brown, D. D., Ed.) pp 683-693, Academic, New York and London.
Walker, J. E., Gay, N. J., Powell, S. J., Kostina, M., & Dyer, M. R. (1987a) *Biochemistry 26*, 8613-8619.
Walker, J. E., Cozens, A. L., Dyer, M. R., Fearnley, I. M., Powell, S. J., & Runswick, M. J. (1987b) *Chem. Scr. 27B*, 97-105.
Wallace, D. C. (1986) *Hospital Practice*, pp 77-92.
Wilbur, W. J., & Lipman, D. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 726-730.
Zeviani, M., Nakagawa, M., Herbert, J., Lomax, M. I., Sherbany, A., Miranda, A., DiMauro, S., & Schon, E. (1987) *Gene 55*, 205-217.

# Control of ATP Hydrolysis by ADP Bound at the Catalytic Site of Chloroplast ATP Synthase As Related to Protonmotive Force and $Mg^{2+}$ †

Ziyun Du and Paul D. Boyer*
*Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024-1570*
*Received July 12, 1988; Revised Manuscript Received September 8, 1988*

ABSTRACT: The activation of the ATP synthesis and hydrolysis capacity of isolated chloroplast membranes by protonmotive force is known to be associated with the release of tightly bound ADP from the ATP synthase. Our data support the view that the activation requires only those structural changes occurring in the steady-state reaction mechanism. The trapping of ADP released during light activation or the chelation of $Mg^{2+}$ with EDTA effectively reduces the rate of decay of the ATPase activity. When the release of tightly bound ADP and $Mg^{2+}$ is promoted by light activation, followed by immediate dilution and washing to retard the rebinding of the ADP and $Mg^{2+}$ released, the ATPase activity remains high in the dark long after the protonmotive force has disappeared. After the addition of ADP and $Mg^{2+}$ the decay of the ATPase activity has the same characteristics as those of the unwashed chloroplast membrane. The results are interpreted as indicating that both $Mg^{2+}$ and ADP must be present prior to exposure to MgATP for the ATPase to be inhibited. However, in contrast to the isolated chloroplast ATPase, the steady-state activity of the membrane-bound ATPase is not inhibited by excess $Mg^{2+}$. The replacement of [$^3$H]ADP from catalytic sites during hydrolysis of unlabeled ATP or during photophosphorylation with unlabeled ADP occurs as anticipated if $Mg^{2+}$ and ADP bound at one catalytic site without $P_i$ block catalysis by all three enzyme sites. The inhibited form induced by $Mg^{2+}$ and ADP may occur only under laboratory conditions and not have an in vivo role.

The activation of the ATPase of chloroplast membranes by exposure to light is a well-established and studied phenomenon (Junge et al., 1970; Schlodder & Witt, 1981; Junesch & Gräber, 1987; Biaudet & Haraux, 1987). This light activation and subsequent inhibition of the ATPase in the dark are known to be closely associated with binding of ADP. A prominent characteristic of the $Mg^{2+}$-activated ATPase of the chloroplast membranes and of the isolated $CF_1$[1] ATPase is the strong inhibition by micromolar concentrations of ADP. Such inhibition has been well documented in important studies from several laboratories (Carmeli & Lifshitz, 1972; Nelson et al., 1972; Strotmann & Bickel-Sandkötter, 1977; Shoshan &

† Supported by Department of Energy Grant DE-FG-03-88ER13845.
* To whom correspondence should be addressed.

[1] Abbreviations: $CF_1$, ATPase portion of the ATP synthase from chloroplasts; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; $P_i$, inorganic phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol.

0006-2960/89/0428-0873$01.50/0 