Tissue-specific expression of the two isoforms of the mitochondrial phosphate carrier in bovine tissues

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Received 15 October 1996

Abstract Comparison of the sequence of the human mitochondrial phosphate carrier (PiC) gene with cDNA clones characterised from a human heart cDNA library suggested the existence of two isoforms of the PiC, which were generated by alternative splicing of exon IIIA or exon IIIB and which differed in 13 amino acids [Dolce et al. (1994) J. Biol. Chem. 269, 10451]. In this work the expression of isoforms A and B of the PiC was investigated in different bovine tissues by Northern blot analysis using two probes that are specific for bovine exon IIIA and exon IIIB, respectively. Isoform A is highly expressed in heart and skeletal muscle. Isoform B is ubiquitously expressed in all tissues that were examined, although at different levels. The tissue-specific expression pattern of the two PiC isoforms is similar to that reported for the isoforms of several mitochondrial proteins required for energy production.

Key words: Phosphate carrier; Isoform; Expression; Transcript; Mitochondria

1. Introduction

The synthesis of ATP during oxidative phosphorylation requires uptake of ADP and phosphate into mitochondria. The translocation of phosphate across the inner mitochondrial membrane is catalysed by a specific transport system known as the phosphate carrier [1]. This carrier catalyses the uptake of phosphate either by proton cotransport or in exchange for hydroxyl ions. The phosphate carrier has been purified and successfully reconstituted into phospholipids [2]. Its primary structure has been determined in various organisms by sequencing cDNA clones encoding the protein [3–6]. The mature protein contains three related internal repeats of about 100 amino acids in length. These repetitive elements are related to those found in the other characterised members of the mitochondrial carrier family, namely the ADP/ATP, oxoglutarate, citrate and carnitine carriers and the uncoupling protein from brown fat [7,8]. Only one gene has been detected for the phosphate carrier in man and cow [3], of which the human one has been localised to chromosome 12q23 [9]. The human and bovine genes have similar structures containing eight introns in equivalent positions [10]. In both organisms two exons named IIIA and IIIB are closely related and they appear to be alternatively spliced. In addition to the close relation of exons IIIA and IIIB in man and cow, evidence of the existence of alternatively spliced forms of the PiC has been found by characterisation of cDNA clones from a human heart cDNA library [10]. The alternative splicing mechanism affects amino acids 4–45 of the mature PiC, which are believed to form the first of the six transmembrane segments of the membrane-embedded protein and to emerge as a large extramembranous loop.

In this report we determine the transcript levels of the two isoforms (A and B) of the PiC in different bovine tissues. We show that isoform A is expressed in large amounts only in heart and skeletal muscle, whereas isoform B is ubiquitously expressed in all tissues investigated, although at different levels.

2. Materials and methods

2.1. Materials

[α-^{32}P]dATP, cDNA synthesis system plus kit and Hybond-N membranes were obtained from Amersham; M13mp19 vector and salmon sperm DNA from Boehringer; pET21b vector from Novagen; Geneclean III kit from Bio 101; and Oligolabelling kit from Pharmacia. The probe for the rat glyceraldehyde 3-phosphate dehydrogenase was a gift of Dr. J.E. Walker. All other reagents were of the highest purity commercially available.

2.2. Amplification of bovine cDNA clones

Bovine poly(A$^+$) mRNAs were prepared from total RNA from heart and liver tissues [11]. Samples of first strand cDNAs, prepared using the cDNA synthesis system plus kit, were employed as template in polymerase chain reactions to amplify the cDNAs encoding the PiC isoforms containing the sequence of exon IIIA (isoform A) and of exon IIIB (isoform B), respectively. The primers used for isoform A corresponded to nucleotides 444–453 linked to 1770–1778 (forward primer) and 5960–5978 (reverse primer) of the bovine PiC gene sequence, and those used for isoform B to nucleotides 444–453 linked to 2051–2059 (forward primer) and 5960–5978 (reverse primer) of the same gene sequence [10]. All primers were synthesised with appropriate linkers. The products of the two PCRs were recovered from agarose gel by the Geneclean procedure and cloned into pET21b expression vector. DNA sequences were determined by the modified dideoxy chain termination method [12].

2.3. Synthesis of probes specific for exon IIIA and exon IIIB

The probes specific for exon IIIA and exon IIIB were amplified by PCR using the expression vector containing the cDNA encoding isoform A or isoform B as template and the following sets of oligonucleotide primers. The primers used to amplify the probe specific for exon IIIA corresponded to 1770–1788 (forward primer) and to 1843–1861 (reverse primer) of the bovine PiC gene sequence, and those used to amplify the probe specific for exon IIIB to 2051–2069 (forward primer) and to 2121–2139 (reverse primer) of the same gene sequence [10]. The products were cloned into M13mp19 vector and sequenced. The fragments with the correct sequence for exon IIIA and IIIB were digested, recovered from an agarose gel, and used as probes in Northern blot analysis.

2.4. Northern blotting

Total RNA was extracted from bovine skeletal muscle, liver, heart, kidney, lung and thyroid by the method of Chirgwin et al. [13]. Samples of total RNA (up to 20 μg) were treated at 50°C for 1 h with 1 M glyoxal in 10 mM sodium phosphate (32 μl), pH 7.0, and then chilled

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Abbreviations: ANT, adenine nucleotide translocator; PiC, phosphate carrier; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate

PII S0014-5793(96)01294-X

in ice water. Dye mix (0.1% bromophenol blue, 1 mM EDTA and 50% glycerol; 4 μl) was added, and the samples were fractionated on an RNase free 1.4% high melting temperature agarose gel submerged in a stirred solution of 10 mM sodium phosphate, pH 7.0. Electrophoresis was carried out at 100 mA for 3.5 h. RNA was transferred to Hybond-N membranes by capillary blotting, and cross-linked to the membrane by UV irradiation at 305 nm for 2 min. The filter was prehybridised at 65°C for 2 h in a solution containing 6×SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate), 5×Denhardt's solution (which contains 1 mg/ml each of poly(vinyl-pyrrolidone), bovine serum albumin (fraction V), and Ficoll), 0.5% (w/v) SDS and boiled sonicated salmon sperm DNA (100 μg/ml). Hybridisations were performed overnight in the same solution containing a labeled probe at a concentration of 2×10^6 cpm/ml. The probes (20 ng) constructed as described above were labeled with [α-^{32}P]dATP (3000 Ci/mmol) using an Oligolabelling kit. The filters were washed four times (15 min each) at 65°C in a solution containing a fixed concentration of SDS (0.1%, w/v), and at decreasing concentrations of SSC from 5× to 0.2×, and were then autoradiographed at −70°C for 8 days with an intensifying screen. For the normalisation of the hybridisation signals an 850 bp probe encoding part of rat glyceraldehyde 3-phosphate dehydrogenase [14] was employed under the same conditions of hybridisation and washing as above.

2.5. Southern blotting

The agarose gels were transferred on Hybond-N membranes by capillary blotting [15]. The filters were hybridised as described for Northern blotting.

3. Results and discussion

Two probes, one specific for the bovine exon IIIA and the other for IIIB, were prepared for a Northern blot analysis of the two transcripts of the PiC gene in various bovine tissues. Since exons IIIA and IIIB are closely related (65.6% identity), we amplified stretches of these exons that were as divergent and long as possible. The probes were 92 nucleotides (probe A) and 89 nucleotides (probe B) in length, and were 60.8% identical (Fig. 1). It was therefore necessary to check their specificity. Fig. 2A,B shows that probe A reacted with the bovine cDNA containing only exon IIIA (lane 2A) and probe B with the bovine cDNA containing only exon IIIB (lane 3B). No cross-reaction was observed between probe A and exon IIIB (lane 3A) and between probe B and exon IIIA (lane 2B). However, as expected, both probes hybridised with the entire human PiC gene (lanes 1A and 1B). In fact, bovine and human exons IIIA have identical nucleotide sequences, and the nucleotide sequences of exons IIIB of these two species differ only for 6 nucleotides out of 122.

Having confirmed that the two probes are absolutely specific, we studied the distribution of the two transcripts arising from alternatively spliced exons IIIA and IIIB of the bovine PiC gene by Northern blot performed on total RNA derived from various bovine tissues, i.e. skeletal muscle, liver, heart, kidney, lung and thyroid. In these experiments the same blot was hybridised with probe A (Fig. 3A), then with probe B (Fig. 3B) and finally with a cDNA encoding part of rat glyceraldehyde 3-phosphate dehydrogenase (Fig. 3C). The transcript containing exon IIIA was expressed very strongly in skeletal muscle and heart (lanes M and H of Fig. 3A). No hybridisation was observed in the lanes corresponding to liver, kidney, lung and thyroid, although autoradiography had been protracted for 8 days. In striking contrast to the specific expression of transcript A in heart and skeletal muscle, the transcript containing exon IIIB was expressed in all the tissues that were examined although at very different levels (Fig. 3B). After 8 days of autoradiography, expression of transcript B was detected most strongly in lung and thyroid, and somewhat lower amounts of its mRNA were found in kidney. The response in liver was very low, and transcript B was only just detectable in heart and skeletal muscle. That these differences in the level of expression of both transcripts A and B in the various tissues tested were not due to gross variations in the amounts of RNAs loaded on the gel was shown in a control experiment with a probe for glyceraldehyde 3-phosphate dehydrogenase RNA (Fig. 3C). It should be noted that with both probes A and B, in addition to the PiC transcript of about 1.5 kb, a weaker hybridisation band estimated to be 4 kb long was observed, as previously reported in a Northern blot of bovine heart poly(A^+) mRNA [3]. The presence of this band, which appeared in all the tissues in about the same ratio with respect to the PiC transcript, cannot yet be explained.

These results clearly demonstrate the existence of two transcripts of the PiC (A and B) generated by alternative splicing of exon IIIA or exon IIIB. Both are present in very different amounts in the various bovine tissues. Furthermore, since the time of autoradiography was the same for the hybridisation of probe A and probe B (Fig. 3), it is apparent that the absolute amounts of PiC are very different from tissue to tissue. For

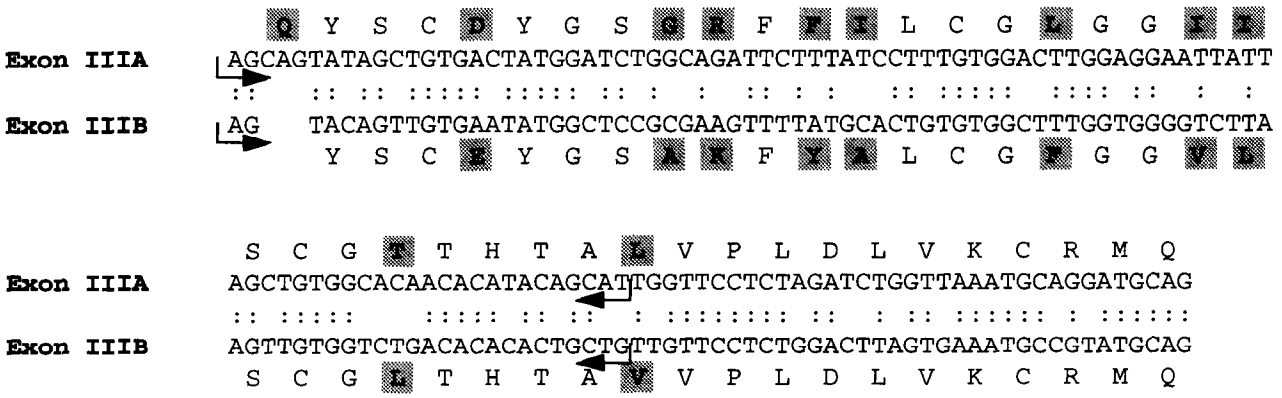

Fig. 1. Nucleotide sequences of probe A and probe B derived for the bovine exon IIIA and exon IIIB, respectively. Exon IIIA and exon IIIB correspond to nucleotides 1770–1894 and to nucleotides 2051–2172 of the bovine PiC gene sequence, respectively. The boundaries of probe A and probe B are denoted by small arrows. The colons indicate identities in the nucleotide sequences; the shaded and bold amino acids differ in the protein sequences.

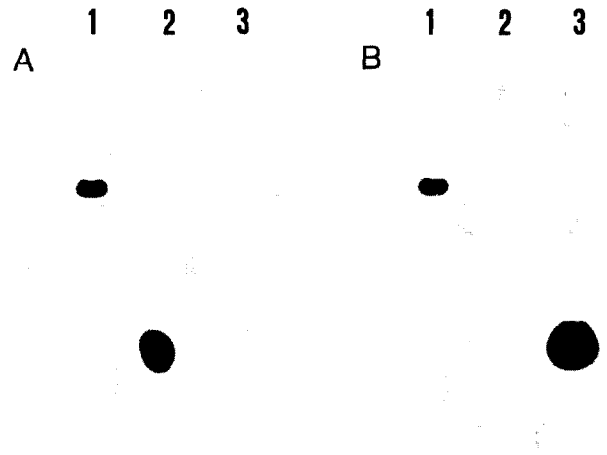

Fig. 2. Hybridisation with probe A and probe B of the entire human PiC gene and of the bovine cDNAs containing either exon IIIA or exon IIIB. Lane 1, 1 μg of the λ12D3 human genomic clone [10] containing the entire PiC gene was digested with the *BglII*; lane 2, 0.5 μg of expression vector pET21b containing the bovine cDNA encoding the PiC isoform A was digested with the restriction enzymes *NdeI* and *EcoRI*; lane 3, 0.5 μg of expression vector pET21b containing the bovine cDNA encoding the PiC isoform B was digested with the restriction enzymes *NdeI* and *EcoRI*. The following probes were employed: panel A, a 92 bp probe corresponding to part of exon IIIA of the bovine PiC gene (probe A); panel B, a 89 bp probe corresponding to part of exon IIIB of the bovine PiC gene (probe B).

example there is much more PiC in heart and skeletal muscle than in liver. Tissue-specific isoforms of several other mitochondrial proteins involved in oxidative phosphorylation have been found. Among these proteins are the mammalian proteolipid subunit of ATP synthase [16], the three mammalian subunits (VIa, VIIa, and VIII) of cytochrome oxidase [17–19], and the human γ-subunit of the ATP synthase complex [20]. The common feature of all these proteins is the presence of two isoforms: a heart type (H), which is abundantly expressed only in muscles, and the liver type (L), which is ubiquitously expressed in all tissues.

Interestingly, the expression pattern of the two isoforms of the PiC resembles that of the mitochondrial proteins listed above, isoform B being the housekeeping form of the PiC and isoform A the form which is specifically present in muscles. The only other mitochondrial translocator which has isoforms (encoded by multiple genes) is the adenine nucleotide translocator (ANT) [21–26]. Of its isoforms, ANT 1 is specific for heart and skeletal muscle, ANT 3 is ubiquitously expressed, and ANT 2 is either absent or minimal in all tissues [27]. The observed tissue-specific differential expression of the PiC isoforms A and B may be used to modulate the rate of ATP production by oxidative phosphorylation for tissue-specific energetic needs. Therefore, the tissue specificity of the two PiC isoforms suggests that they are differently regulated and/or have different kinetic properties that could account for the differential reliance of the tissues where they are present on oxidative phosphorylation. It is known that certain ener-

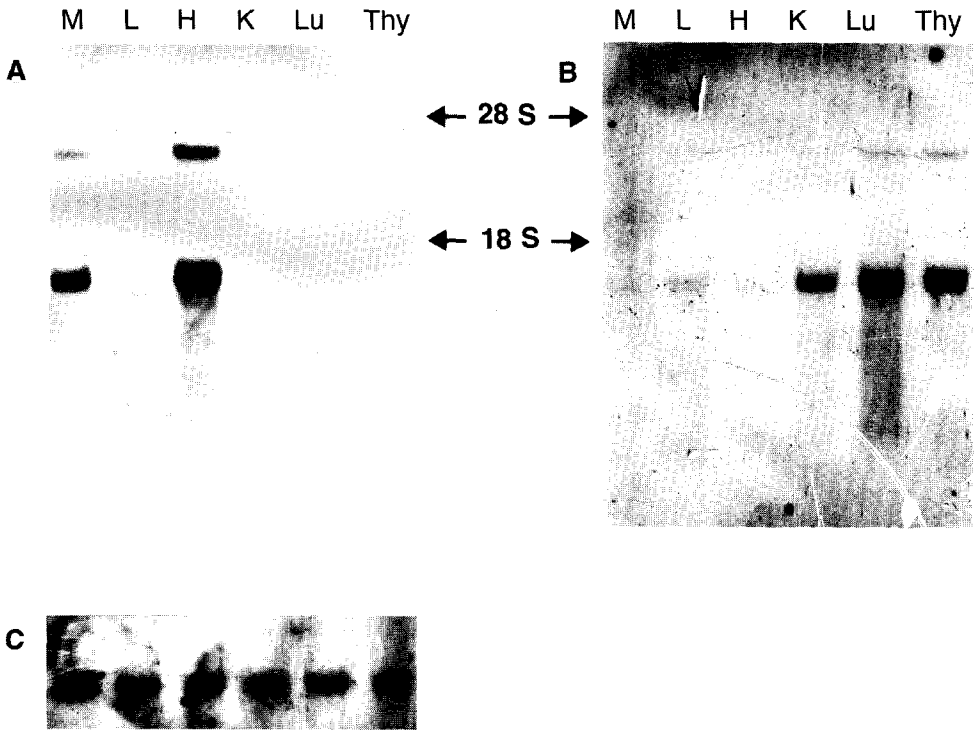

Fig. 3. Expression of the isoforms A and B of the PiC in bovine tissues analysed by Northern blot analysis. The following probes were employed: panel A, a 92 bp probe corresponding to part of exon IIIA (probe A); panel B, a 89 bp probe corresponding to part of exon IIIB (probe B); panel C, a 0.85 kb probe from a rat cDNA encoding glyceraldehyde 3-phosphate dehydrogenase. The following RNA samples were used: M, skeletal muscle; L, liver; H, heart; K, kidney; Lu, lung; Thy, thyroid. About 20 μg of total RNA was loaded in each slot. Between panels A and B the positions of 28S and 18S ribosomal RNAs are shown (4800 and 1900 nucleotides, respectively). After 8 days of autoradiography at −70°C, blot A was stripped by boiling in 0.1% (w/v) SDS for 3 min, and rehybridised with probe B. After a further 8 days of autoradiography at −70°C, blot B was subjected to the procedure described before and then hybridised again with the glyceraldehyde 3-phosphate dehydrogenase probe.

getic parameters, e.g. the ADP/ATP ratio, the cytosolic inorganic phosphate concentration and the mitochondrial membrane potential, are different in heart and liver [28,29]. Moreover, two processes of interest take place in liver and not in heart: a substantial amount of ATP is split into ADP and inorganic phosphate within the mitochondrial matrix for the synthesis of urea, and, secondly, inorganic phosphate can also be taken up by mitochondria via the dicarboxylate carrier.

Our studies suggest that the housekeeping PiC isoform B matches the basic energy requirements of all tissues (being expressed constitutively, although at different levels) and isoform A becomes operative to accommodate the higher energy demands associated with contraction of striated muscle fibers. Thus we hypothesise that during muscle contraction the capacity of isoform B (predicted to have a higher affinity for phosphate) will be overwhelmed, and isoform A (predicted to have a lower affinity for phosphate) will be activated and regulated by the increasing concentrations of available substrate.

Acknowledgements: This work was supported by the Consiglio Nazionale delle Ricerche, Target Project 'Ingegneria genetica' and by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

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