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Characterization of two different genes (cDNA) for cytochrome *c* oxidase subunit VIa from heart and liver of the rat

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By antibody screening of a rat liver and a rat heart cDNA library in λgt11 two clones coding for the liver- and heart-specific subunit VIa of rat cytochrome *c* oxidase were isolated. In the heart cDNA sequence a TAA stop codon was found in frame 18 bp 5′ upstream of the first methionine codon, thus excluding a leader sequence for this protein. The two cDNAs contain the full-length coding region of two subunits. The amino acid sequences of the two subunits show only 50% homology, whereas 74% homology was found between rat heart and bovine heart subunit VIa. By Northern blot analysis it is shown that the gene for subunit VIa from heart is only expressed in heart and skeletal muscle, whereas that from liver is also expressed in kidney, brain, heart and weakly in muscle.

Key words: amino acid sequence homology/cDNA/cytochrome *c* oxidase/nucleotide sequence homology/tissue-specific expression

Introduction

Extraordinary features make cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, of particular interest among multi-subunit enzyme complexes of eukaryotic cells: (i) it is composed of multiple subunits encoded on two different genetic systems—the mitochondrial and the nuclear genome; (ii) the number of subunits is variable, increasing with the evolutionary stage of the organism; (iii) the assembly of the transmembranous enzyme complex from both sides of the inner mitochondrial membrane involves polypeptides with and without leader sequences; (iv) whereas the catalytic subunits encoded by mitochondrial DNA are identical in all complexes of an organism, the nuclear-encoded subunits occur in multiple tissue- and developmental-specific isoforms (for review see Kadenbach *et al.*, 1987); (v) recently a number of human genetic diseases have been described (mitochondrial myopathies, see DiMauro *et al.*, 1987, for review), based on defective cytochrome *c* oxidase.

The tissue-specific occurrence of nuclear-encoded subunits was originally discovered by different apparent mol. wts of some subunits on SDS–PAGE (Merle and Kadenbach, 1980) and was later corroborated by different immunological relationships (Jarausch and Kadenbach, 1982; Kuhn-Nentwig and Kadenbach, 1985), different N-terminal amino acid sequences of corresponding subunits from liver and heart of the same organism (Kadenbach *et al.*, 1982, 1983), different binding domains for cytochrome *c* (Kadenbach and Stroh, 1984) and different distribution of SH-groups in nuclear-encoded subunits (Stroh and Kadenbach, 1986). The functional role of each tissue-specific subunit of cytochrome *c* oxidase is unknown. It was assumed, however, that they regulate the rate of respiration and efficiency of energy transduction according to the variable needs in different tissues (Kadenbach, 1986). This hypothesis was corroborated by different kinetic properties of isolated cytochrome *c* oxidase from bovine liver and heart (Merle and Kadenbach, 1982; Büge and Kadenbach, 1986).

In addition to mammalian cytochrome *c* oxidase, which contains always 13 subunits (Kadenbach *et al.*, 1987), isozymes have also been found for cytochrome *c* oxidase from *Dictyostelium discoideum* (Bisson and Schiavo, 1986), which is composed of only eight subunits, and from *Saccharomyces cerevisiae*, which contains nine subunits (Cumsky *et al.*, 1985). In yeast, only subunit V was found to occur in two isoforms and the two genes were recently characterized (Cumsky *et al.*, 1987). Whereas the mammalian isozymes are kinetically different, the two yeast isogenes for subunit V do not differ significantly in their ability to assemble and to function as subunits of the holoenzyme (Trueblood and Poyton, 1987).

Apart from some N-terminal amino acid sequences, up to now no complete sequences of two tissue-specific 'isopeptides' of the mammalian enzyme have yet been reported. Here we present for the first time, two different cDNAs for subunit VIa of rat liver and heart cytochrome *c* oxidase, which show only 50% homology in their deduced amino acid sequences. The heart subunit VIa, although having a transmembraneous sequence of 20 hydrophobic amino acids, does not contain a leader peptide.

Results

Screening of a rat liver cDNA library in λgt11 with affinity-purified monospecific antisera to rat liver cytochrome *c* oxidase subunit VIa revealed three positive clones. The apparent insert sizes were 200 bp (clone RL-1), 500 bp (clone RL-2) and 500 bp (clone RL-3) respectively. After subcloning in M13mp9, both strands of the three cDNAs were sequenced by the Sanger dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The insert of clone RL-2 showed no homology of its deduced amino acid sequence to the known N-terminal sequence of any of the 10 nuclear-encoded subunits of the rat liver enzyme (Ungibauer, 1987) or of the bovine heart enzyme (see Kadenbach *et al.*, 1987). The insert of clone RL-1 contained a sequence of only 134 bp which harboured the codons of 10 C-terminal amino acids of subunit VIa, as eight of these residues were identical to those of the bovine heart amino acid sequence (Meinecke and Buse, 1985). The third clone

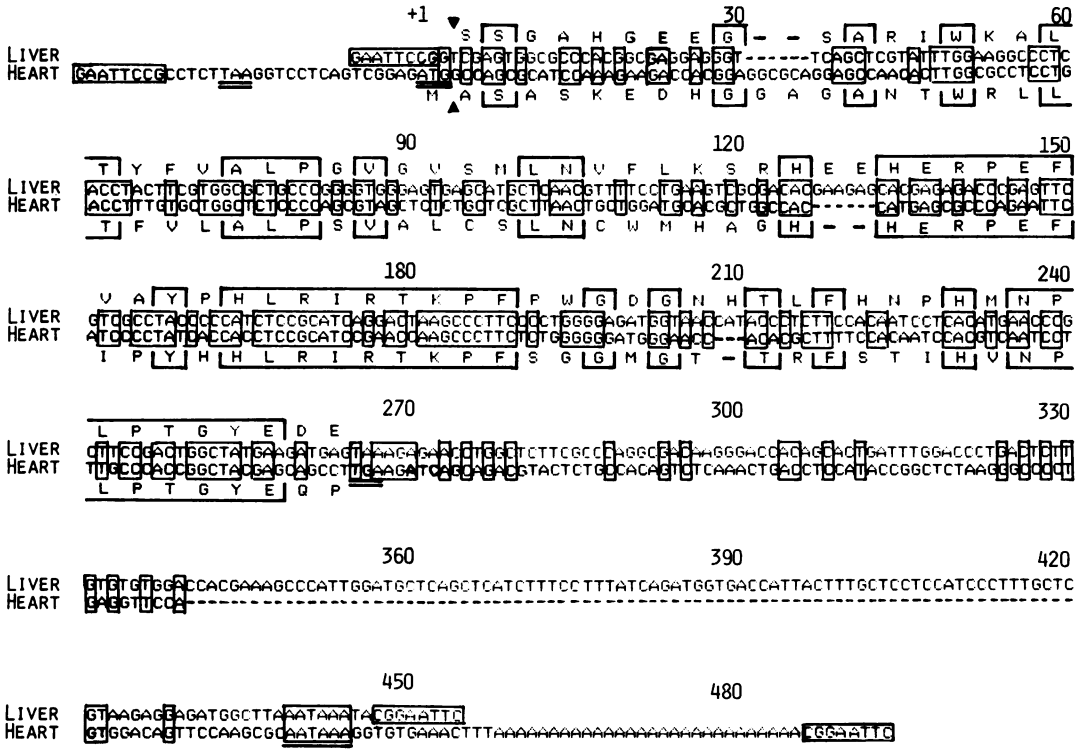

Fig. 1. cDNA and deduced amino acid sequences of two isogenes coding for subunit VIa of rat heart and rat liver cytochrome *c* oxidase. Linker sequences used to construct the cDNA libraries, as well as homologous nucleotides are boxed. Dashes indicate insertions in both nucleotide and protein sequences. Start and termination codons as well as polyadenylation signals are double underlined. Amino acid sequence homologies are in large boxes. The first amino acid of the mature protein precedes an arrowhead.

(RL-3) contained an insert of 438 bp, comprising the entire protein coding and the 3′-non-coding region of subunit VIa mRNA (Figure 1). In this cDNA sequence nucleotides 234−367 were identical to those of clone RL-1.

Because no antiserum to subunit VIa of heart cytochrome *c* oxidase was available, the rat heart cDNA library was screened with a mixture of affinity-purified antisera to rat liver and human heart subunit VIa and to bovine heart holoenzyme. From six positive clones, which had apparent insert sizes of 440 bp (clone RH-1), 450 bp (clone RH-2), 450 bp (clone RH-3), 1700 bp (clone RH-4), 1800 bp (clone RH-5) and 100 bp (RH-6) respectively, four had an internal *Eco*RI restriction site (RH-1, RH-2, RH-3 and RH-4). Clones RH-1 and RH-2 were subcloned in M13mp9 and both strands were sequenced according to Sanger *et al.* (1977). The complete sequence of the two *Eco*RI fragments of clone RH-1 is presented together with that of the liver clone RL-3 in Figure 1. The two cDNA sequences as well as their deduced amino acid sequences are aligned in order to obtain maximal homology.

In the heart cDNA an additional sequence of 6 bp is found in the coding region. In the liver cDNA two additional sequences of 6 bp and 3 bp are found in the coding region and one additional sequence of 81 bp in the 3′-non-coding region. Therefore the mature polypeptide (see below) in liver contains an additional amino acid (85 as compared to 84 in the mature polypeptide of heart). From the homology of the deduced N-terminal amino acid sequence of the heart cDNA with the mature bovine heart amino acid sequence (Meinecke and Buse, 1985, see Figure 4), we assume that the mature rat heart amino acid sequence begins after the first methionine, which apparently is cleaved off post-translationally. This assumption is further substantiated by the occurrence of a TAA stop codon in frame 18 bp upstream of the first methionine codon. Because this TAA stop codon was also found in clone RH-2, which is 5 bp shorter at the 5′ end (not shown), we conclude that rat heart subunit VIa is synthesized without a cleavable precursor sequence.

The liver cDNA contains also the polyadenylation signal AATAAA but is missing the poly(A)$^+$ tail. In addition, the 5′ non-coding region is missing. Nevertheless it contains the complete sequence of the coding region for the mature polypeptide. This follows from the deduced N-terminal amino acid sequence, which is identical to the N-terminal sequence of mature rat liver subunit VIa (SSGAHG), as determined by N-terminal amino acid sequencing of the isolated polypeptide (Ungibauer, 1987).

The aligned two cDNA sequences show very little homology in the 3′-non-coding region and only 56% homology in the coding region (Figure 1). In 27 of the 42 codons for conserved amino acids, as well as in the stop codon, the third base differs, and in three of these codons the first base differs in addition. The maximal homology is found in a stretch of nine homologous amino acids, where two boxes of nine homologous base pairs occur. From these results a very long period of independent evolution of the gene for subunit VIa from liver and heart is evident.

The two deduced amino acid sequences show only 50% homology. But both polypeptides must be tightly associated in the functional enzyme complex with three identical mitochondrial encoded subunits and probably a further seven identical and two variable nuclear-encoded subunits. Therefore some homologous structure must occur in both polypeptides which accomplishes their tight binding. In

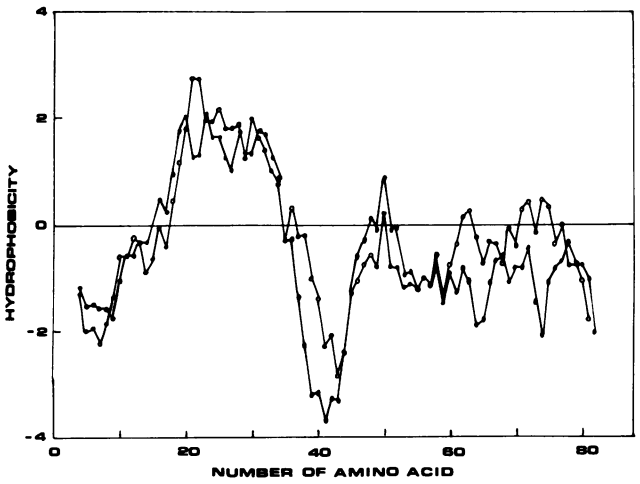

Fig. 2. Hydropathy plot of rat liver and rat heart subunit VIa amino acid sequences. The method of Kyte and Doolittle (1982) was applied. Each point represents the average hydrophobicity for a sequence of seven amino acids. ○– – ○ rat heart; ●– – ●, rat liver subunit VIa.

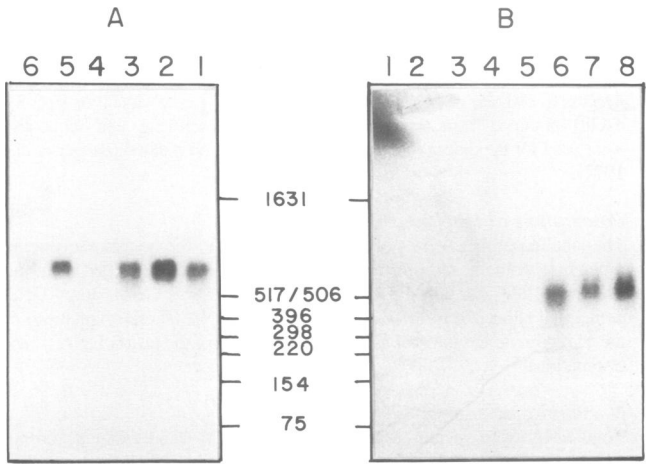

Fig. 3. Northern blot analysis of total RNA and poly(A)$^+$ RNA from rat heart, liver, kidney, brain and skeletal muscle with probes of rat liver and heart subunit VIa of cytochrome *c* oxidase. (**A**) Hybridization with liver probe. **Lane 1**, 3 μg heart poly(A)$^+$ RNA; **lane 2**, 3 μg liver poly(A)$^+$ RNA; **lane 3**, 3 μg kidney poly(A)$^+$ RNA; **lane 4**, no addition; **lane 5**, 15 μg brain total RNA; **lane 6**, 15 μg skeletal muscle total RNA. (**B**) Hybridization with heart probe. **Lane 1**, 15 μg brain total RNA; **lane 2**, 3 μg kidney poly(A)$^+$ RNA; **lane 3**, 15 μg kidney total RNA; **lane 4**, 15 μg liver total RNA; **lane 5**, 3 μg liver poly(A)$^+$ RNA; **lane 6**, 3 μg heart poly(A)$^+$ RNA; **lane 7**, 15 μg skeletal muscle total RNA; **lane 8**, 15 μg heart total RNA. The numbers in the middle indicate the position of nucleotide mass marker (pBR 322 DNA fragments obtained after *Hin*fI digestion).

Figure 2 the hydropathy plot of both subunits is presented as calculated by the method of Kyte and Doolittle (1982) with a rolling average of seven amino acid residues. The two plots are very similar except that the liver subunit VIa is clearly more hydrophilic around amino acid number 40 and in the C-terminal part of the sequence. Both subunits show a sequence of 20 hydrophobic amino acids (positions 16–36), characteristic of a membrane-spanning polypeptide.

In order to explore tissue-specific expression of the liver and heart subunit VIa, a Northern blot analysis of RNA from different rat tissues was performed (Figure 3). The labelled probes were synthesized according to the Sanger sequencing procedure without addition of dideoxynucleotides with the full-length M13mp9 clone from liver and the M13mp9 clone from heart which comprises the 5′ region up to the internal *Eco*RI restriction site at position 145. With the heart probe, only RNA from heart and skeletal muscle each gave a single hybridization signal of ~520 bp. No signal was found with RNA from liver, kidney and brain, clearly indicating the specific expression of the heart gene for subunit VIa in heart and skeletal muscle.

In contrast to the heart-specific probe, the liver-specific probe hybridized with a single RNA band each of liver, kidney, brain, heart and skeletal muscle. Since the apparent sizes of these signals are all larger than those obtained in heart and muscle with the heart probe, we conclude that a specific hybridization of the liver probe has also occurred; but in contrast to the heart gene, the liver gene appears to be expressed in a less specific way. The larger size of the liver mRNA is obviously due to its larger 3′-non-coding region (see Figure 1). In order to prove that the hybridization signals of the liver probe obtained with RNA from heart and skeletal muscle are not due to cross-hybridization with the heart-specific mRNA, the washing temperature was increased from 52°C to 65°C. The same relative intensity of signals with RNA from different tissues was obtained (not shown), indicating that the 'liver-specific' gene is also expressed in heart and muscle.

Discussion

In this paper the cDNA sequences of two tissue-specific isogenes for a subunit of mammalian cytochrome *c* oxidase are presented for the first time. From N-terminal amino acid sequences at least two other subunits (VIIa and VIII) were concluded to occur in different forms in the liver and heart enzyme (Kadenbach *et al.*, 1982, 1983; Capaldi, 1988). Whereas only 50% homology is found between the rat liver and rat heart sequence (42 homologous amino acids out of 84 in heart and 85 in liver), a homology of 75% is found between the rat heart and bovine heart amino acid sequence (Meinecke and Buse, 1985), thus corroborating our previous conclusion on subunits of cytochrome *c* oxidase isozymes based on immunological data (Jarausch and Kadenbach, 1982): tissue specificity overrides species specificity.

In two independent clones of the rat heart cDNA a TAA stop codon is found in frame 18 bp upstream of the methionine start codon, thus excluding a precursor sequence for this protein. A similar situation was found in the cDNA of rat liver subunit VIc, where also a stop codon (TAG) was found in frame 18 bp 5′ upstream of the methionine start codon (Suske *et al.*, 1987). Both subunits (VIa and VIc) contain a stretch of 20 hydrophobic amino acids, suggesting a transmembraneous disposition. But whereas the N-terminal sequence of subunit VIc resembles a mitochondrial leader sequence (lack of acidic and presence of basic and hydrophobic amino acids and two serines)—which, however, is not cleaved (Suske *et al.*, 1987)—subunit VIa from heart contains two and that from liver contains one acidic amino acid in its N-terminal sequence. Such residues are usually absent in mitochondrial leader sequences (see Nicholson and Neupert, 1987 for review). From studies on the accessibility of rat liver subunit VIa from both sides of the mitochondrial membrane by proteases, a membrane-spanning region was postulated (Jarausch and Kadenbach, 1985). We must

therefore assume that subunit VIa is transported into mitochondria without a N-terminal leader sequence as has been found for other inner membrane proteins: the ADP/ATP carrier (Zimmermann *et al.*, 1979; Hatalova and Kolarov, 1983), the uncoupling protein of brown adipose tissue (Bouillaud *et al.*, 1986; Ridley *et al.*, 1986), two subunits of the bcl complex of yeast (van Loon *et al.*, 1983) and the ubiquinone-binding protein of the bcl complex from bovine (Nishikimi *et al.*, 1986).

From mapping studies of the cytochrome *c* binding domain of cytochrome *c* oxidases from different tissues, carboxylic groups of subunit VIa from liver and kidney but not from heart and skeletal muscle were found to participate in the binding of cytochrome *c* (Kadenbach and Stroh, 1984; Kadenbach *et al.*, 1986). The four additional negative charges of the liver as compared to the heart subunit (Figure 1) appear to function in the modified binding of cytochrome *c*, as manifested in the different kinetics of cytochrome *c* oxidation of the liver and heart enzyme (Merle and Kadenbach, 1982; Büge and Kadenbach, 1986).

From the Northern blot analysis (Figure 3) it clearly follows that the gene for subunit VIa from heart is exclusively expressed in heart and skeletal muscle. This gene is apparently not expressed in liver, kidney and brain of adult rats. The specificity of expression of the liver gene is apparently less exclusive. The data suggest that two different cytochrome *c* oxidase isozymes are expressed in heart which differ in subunit VIa.

The apparent lack of cross-hybridization between the gene for subunit VIa from liver and heart suggests that probes of the two genes cannot be used to identify the corresponding isogene from other tissues by hybridization methods. Therefore the term 'gene family' may not apply to these evolutionary distant genes. The suggestion of Bachman *et al.* (1987) that only one gene for subunit IV occurs in the bovine genome may be incorrect, because a distantly related gene may not have been detected by their Southern blot analysis.

Two different genes encoding the bovine mitochondrial ATP synthase proteolipid were described by Gay and Walker (1985). The tissue-specific expression of the two genes, however, results in the same mature functional proteolipid.

The data presented in this paper prove for the first time the occurrence of multiple functional genes for homologous nuclear-encoded subunits of cytochrome *c* oxidase. The developmentally specific expression of a defective muscle-specific subunit of the enzyme is assumed to represent the molecular basis of fatal infantile mitochondrial myopathies, characterized by defective cytochrome *c* oxidase (for review see DiMauro *et al.*, 1987).

Materials and methods

Libraries and enzymes

cDNA libraries from rat liver and rat heart were obtained from Clontech (Heidelberg). Restriction and DNA modification enzymes were purchased from Boehringer (Mannheim) and Medac (Hamburg). T7 polymerase sequencing kit (Sequenase) was obtained from Renner GmbH (Dannstadt).

Screening of λgt11 cDNA libraries with antibodies

Antisera to rat liver subunit VIa, human heart subunit VIa and bovine heart holocytochrome *c* oxidase were raised in rabbits as previously described (Merle *et al.*, 1981). Antibodies were affinity purified on a column of Sepharose 4B conjugated with rat liver cytochrome *c* oxidase, or on bands of nitrocellulose sheets containing blotted rat heart subunit VIa, separated by SDS–gel electrophoresis. The antibodies were eluted from columns with 1 M glycine–HCl, pH 2.5, and from sheets with 0.2 M glycine–HCl, pH 2.5, and were immediately neutralized with 2 M Tris–HCl, pH 10.0.

Recombinant phages from the rat liver or rat heart cDNA library were plated on a lawn of *Escherichia coli* Y 1090 to a density of ~25 000 plaques/150-mm plate (six plates each) and incubated at 42°C for 3 h. A dry nitrocellulose filter (Schleicher and Schüll, BA 85), previously saturated with 10 mM isopropyl-β-D-thiogalactoside, was overlaid. After incubation for 6–8 h at 38°C, filters were removed, washed twice in PBS (10 mM NaP_i, pH 7.4, 150 mM NaCl) and saturated with 1% bovine serum albumin (BSA) in PBS by incubation for 30 min. Six filters of each library were then rocked for 2–4 h at room temperature in 40 ml PBS containing 1% BSA and the affinity-purified antibodies from ~0.5 ml antiserum to rat liver subunit VIa (liver library) or from 0.75 ml of four mixed antisera to rat liver subunit VIa, 0.5 ml antiserum to human heart subunit VIa and 0.5 ml antiserum to bovine heart holoenzyme (heart library). After several washings in PBS containing 0.2% Triton X-100 the filters were incubated with the second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate from BioRad, Western blotting grade, affinity purified) diluted 1/2000 in PBS containing 1% BSA for 1 h at room temperature. After several washings in PBS, 0.2% Triton X-100, the filters were incubated twice (5 and 10 min) with staining buffer (0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$). Staining under reduced light or in the dark was performed by incubation with 0.33 mg/ml NBT (nitroblue tetrazolium from Sigma) and 0.167 mg/ml BCIP (5-bromo-4-chloro-3-indolylphosphate from Sigma) in staining buffer (7.5 ml/100-cm^2 filter) for up to 3 h until blue plaques were visible. Stock solutions of NBT (75 mg/ml in 70% dimethylformamide) and BCIP (50 mg/ml in 100% dimethylformamide) were kept at 4°C in the dark. Positive clones were purified by rescreening under reuse of the antibody solutions.

DNA sequence analysis

For analysis of the nucleotide sequence the cDNA inserts were subcloned into the *Eco*RI site of M13mp9 in both directions. [α-^{32}P]dATP or [α-^{35}S]-dATP (obtained from Amersham Buchler, Braunschweig) and Sequenase were used for the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Preparation of labelled probes

The labelled probes were synthesized according to the Sanger sequencing procedure without addition of dideoxynucleotides as described (Hu and Messing, 1982) using [α-^{32}P]dATP. The template was the M13mp9 DNA containing either the inserted full-length liver cDNA (sense orientation) or the 5′ region up the internal *Eco*RI restriction site of the heart cDNA (sense orientation).

Northern blot analysis

Total RNA from various tissues of the rat was isolated either according to Cathala *et al.* (1983) or by the protocol of Amersham's RNA extraction kit. Poly(A)$^+$ RNA was obtained after passing through an oligo d(T)–cellulose column.

Fifteen micrograms of total RNA or 3 μg poly(A)$^+$ RNA were separated on a 1.5% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose filters according to standard procedures (Maniatis *et al.*, 1982). Prehybridization was carried out in 5 × NaCl/Cit, 5 × Denhardt's solution, 25 mM sodium phosphate, pH 6.4, 0.1% SDS, 250 μg/ml sonicated herring sperm DNA, 25 μg/ml poly(A), 50% formamide at 42°C for 4 h. Hybridization with labelled probes from liver and heart (2.4×10^6 c.p.m.) was carried out in 10 ml prehybridization solution at 42°C for 20 h. The filters were washed 4 times for 15 min with 2 × NaCl/Cit, 0.1% SDS at 52°C (liver and heart probe) or at 65°C (liver probe).

Acknowledgements

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