Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7246–7250, October 1988
Cell Biology

Two transcripts encode rat cytochrome b_5 reductase

(cDNA/erythrocytes/liver/membrane proteins/RNase A mapping)

GRAZIA PIETRINI, PAOLA CARRERA, AND NICA BORGESE

Consiglio Nazionale delle Ricerche, Center for Cytopharmacology, University of Milan, 20129 Milan, Italy

Communicated by George E. Palade, June 6, 1988

ABSTRACT **A cDNA expression library in λgt11 was screened with affinity-purified polyclonal anti-rat cytochrome b_5 reductase antibodies. One positive clone out of 450,000 clones was isolated and found to be incomplete. This clone was used to rescreen the library, and a second, overlapping clone that contained the entire coding sequence was isolated. RNA gel blots showed that the two overlapping clones contained ≈90% of the reductase mRNA sequence. Sequencing data showed (*i*) that rat reductase has a 93% sequence similarity with bovine and human reductase and (*ii*) that reductase is not synthesized as a high molecular weight precursor. Results of Southern blot analysis were consistent with the hypothesis that a single gene codes for the soluble and membrane-bound (microsomal and mitochondrial) forms of the reductase, present in erythrocytes and liver, respectively. The cloned cDNA was used to study reductase transcripts in liver and reticulocytes. Two antisense RNA probes that together covered the entire coding region and part of the noncoding region of reductase mRNA were used in RNase A protection experiments. These probes detected only one transcript in liver, suggesting that endoplasmic reticulum and mitochondrial reductase are translated from the same mRNA. In contrast, two transcripts were detected in reticulocytes, one of which mismatched the liver probe ≈30 nucleotides downstream from the initiation codon. Since the soluble and membrane form of the reductase are known to differ at the N terminus, we suggest that this second transcript encodes soluble reductase.**

NADH-cytochrome b_5 reductase (NADH:ferricytochrome-b_5 oxidoreductase, EC 1.6.2.2) is an enzyme that exists as an integral membrane component, on endoplasmic reticulum and outer mitochondrial membranes of liver (1, 2) and many other tissues (3), and as a soluble protein, in erythrocytes (4). Genetic studies in man suggest that the membrane-bound and soluble forms are products of the same gene (5).

Structural studies on the membrane-bound and soluble forms of the reductase in a variety of species have demonstrated (*i*) that the membrane-bound form (300 amino acids) consists of a large cytoplasmic domain (≈275 amino acids) and a short N-terminal membrane anchor that contains a myristic acid covalently attached to the N-terminal glycine (6) and (*ii*) that the soluble form, purified from human erythrocytes, has a primary structure identical to the cytoplasmic domain (comprising the 276 carboxyl-terminal amino acids) of its membrane-bound counterpart (7, 8). Thus, it has been suggested that the soluble form is derived from the membrane enzyme by posttranslational proteolysis during the maturation of erythroblasts (4, 8).

We have been interested in elucidating the biogenetic relationships between the differently localized reductase forms. Studies in rat liver have demonstrated (*i*) that the membrane-bound form is synthesized on free polysomes (9) and inserted posttranslationally and independently into endoplasmic reticulum and outer mitochondrial membranes (10, 11) and (*ii*) that the mitochondrial and endoplasmic reticulum forms cannot be distinguished by peptide mapping or immunological criteria (12). These data suggested that an identical protein is targeted independently to two membrane systems. In addition, our work on the rat soluble enzyme showed that its apparent molecular weight was very close to that of its membrane-bound counterpart (13), suggesting that proteolytic processing might not be the mechanism responsible for its generation. In the present study, we have carried our investigation further by studying the transcripts encoding cytochrome b_5 reductase in two cell types—liver cells and reticulocytes—that predominantly contain the membrane-bound and soluble reductase, respectively. To this purpose, we have cloned a cDNA containing the entire reductase coding sequence* that we have used as probe in RNA gel blotting and RNase A protection experiments.

MATERIALS AND METHODS

General. Most of the methods used in this study are described in Maniatis *et al.* (14). Recombinant λgt11 phage DNA was prepared from lysogenous host bacteria as described by Davis *et al.* (15). DNA fragments, purified by agarose gel electrophoresis, to be used as probes for plaque hybridization or for RNA gel or Southern blots were labeled by the hexanucleotide random-priming method (16). DNA sequencing was carried out by the dideoxy chain-termination method (17) by using the kits of Promega Biotec (Madison, WI) or Amersham for double-stranded (pGEM-3) or single-stranded (M13) recombinant vectors, respectively. RNA was prepared by the guanidine isothiocyanate/CsCl procedure (18) from livers or reticulocyte-enriched preparations (see below) obtained from male Sprague–Dawley rats [150–200 g (body weight)]. Poly(A)$^+$ RNA was purified from total RNA by poly(U) paper affinity chromatography (19) by using Hybond mAP (Amersham).

Immunological Screening of the cDNA Library. A rat liver cDNA expression library in λgt11 was kindly provided by M. Mueckler (20). Rabbit affinity-purified anti-rat cytochrome b_5 reductase antibodies (2), preadsorbed with lysates of plating bacteria (*Escherichia coli* Y1090), were used for immunological screening of the library as described by Huynh *et al.* (21), except for radioimmunostaining of the nitrocellulose replicas that was performed under the same conditions as those used for immunoblotting in our laboratory (2, 13).

Rat Reticulocyte-Enriched Preparation. Male Sprague–Dawley rats, made anemic by phenylhydrazine treatment as described (13), were sacrificed by decapitation. The blood was collected into a beaker containing 0.1 M sodium phosphate and 50 mM EDTA (pH 7) and passed through a microcrystalline cellulose-α-cellulose column (Sigma). We

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: nt, nucleotide(s).

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03867).

have found that this procedure, which is known to remove leukocytes from human blood (22), is effective with rat blood as well [removal of 96% of the leukocytes (23)]. The erythrocyte preparation was then washed twice with isotonic phosphate-buffered saline to remove plasma, and the percentage of reticulocytes in the final preparation was determined on smears after staining with brilliant cresyl blue. Between 56% and 67% of the erythrocytes in these preparations were reticulocytes.

Single-Stranded RNA Probes. The recombinant DNA isolated from the library by plaque hybridization yielded two fragments of ≈500 and ≈900 base pairs (bp), respectively, after *Eco*RI digestion (see *Results*). These fragments were inserted into the *Eco*RI site of pGEM-3 (Promega Biotec), yielding plasmids pG500 and pG900, respectively. Depending on the orientation of the insert, these plasmids generated sense or antisense transcripts, when transcribed from the T7 promoter. The templates were truncated at sites downstream from the cloned insert relative to the T7 promoter but within the vector polylinker region to yield linear molecules: pG500 with *Xba* I, and pG900 with *Bam*HI. pG500 was also truncated at an internal site of the insert DNA with *Ava* I [139 nucleotides (nt) from the 5′ end]. The linearized templates were used for the synthesis of high-specific-activity [^{32}P]GTP-labeled RNA (24). The labeled RNA was purified by DNase treatment and then by phenol extraction and ammonium acetate/ethanol precipitation (25).

RNase A Protection Experiments. The RNase protection procedure was performed as described by Zinn *et al.* (26) with a mixture of RNase A and T1 to digest unhybridized probe. Protected probe fragments were analyzed on 6% sequencing gels. Labeled RNA molecular size markers were synthesized from the Riboprobe Gemini positive control template (Promega Biotec) by using SP6 and T7 polymerase.

RESULTS

Cloning and Sequence Analysis of Rat Liver Cytochrome b_5 Reductase cDNA. To search for a cDNA clone specifying cytochrome b_5 reductase, a rat liver cDNA library in λgt11 (20) was screened with polyclonal antireductase antibodies. Out of 450,000 plaques, 1 plaque gave a clearly positive signal. Digestion of the purified recombinant DNA with *Eco*RI released a 1500-bp fragment, which was purified and subcloned into pGEM-3. Partial sequence analysis of this clone (referred to as the first clone) revealed at one of its extremities a reading frame that corresponded to a sequence identical to amino acids 140–159 of bovine liver microsomal reductase (6). The other extremity of the clone did not contain a poly(A) tail. These data, although establishing the identity of our clone, also demonstrated that it was lacking the 5′-terminal portion of the coding region as well as the 3′ terminus of reductase cDNA (Fig. 1).

To search for a clone containing the entire coding sequence for the reductase, the 5′-terminal *Eco*RI–*Pvu* II fragment of the first clone (see Fig. 1) was used to rescreen the λgt11 library by plaque hybridization. Six positive clones out of 450,000 plaques were detected. Three of these clones released two fragments upon *Eco*RI digestion, indicating that an internal *Eco*RI site was present in reductase cDNA. One of these clones (referred to here as the second clone), consisting of two fragments of ≈900 and ≈500 bp, was selected for further analysis. The two fragments were subcloned separately into pGEM-3 to obtain plasmids pG900 and pG500, respectively, and subjected to restriction mapping and sequence analysis. As shown in Fig. 1, the ≈900-bp fragment was found to correspond to the 5′-terminal ≈900 nt of the first clone, whereas sequence analysis of the ≈500-bp fragment revealed that it contained the initiator codon of reductase mRNA (see the sequence analysis below).

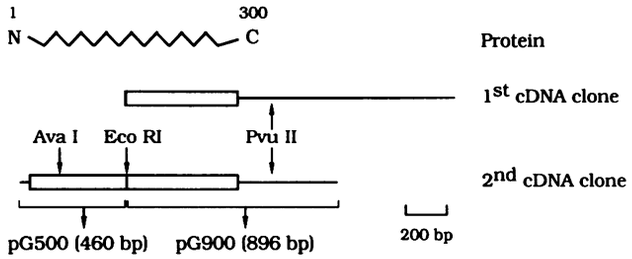

FIG. 1. Relationship between the two reductase cDNA clones isolated in this study and the encoded protein. The insert isolated from the first clone specified the C-terminal portion of the reductase and contained in addition ≈1000 noncoding nucleotides. The second overlapping clone contained the entire coding sequence. This clone contained an internal *Eco*RI site. The two fragments released from λgt11 by *Eco*RI digestion were subcloned separately into pGEM-3 to obtain clones pG500 and pG900. The open box represents the coding region, and the single line represents the noncoding regions of the clones. The positions of the *Pvu* II and *Ava* I sites are indicated.

The sequence of the entire second clone (≈500-bp plus ≈900-bp fragments) is shown in Fig. 2. An AUG codon at 34 nt from the 5′ terminus of the clone, placed within the consensus context for initiation (27), was followed by an open reading frame corresponding to a protein with 93% similarity to the bovine liver protein (6) and to the portion of the human protein whose sequence is known (8). A termination codon at position 1, in frame with the AUG at position 34, ruled out the possibility of alternative initiation sites in liver reductase mRNA, upstream to position 1 of our clone.

The cloned cDNA was used as probe to determine the size of reductase mRNA in liver and reticulocytes by RNA gel blotting (Fig. 3). The probe hybridized to a ≈2200-nt species in liver and to a slightly smaller RNA (2050 nt) in reticulocytes. Thus, the two clones that we isolated contained ≈90% of the sequence of liver reductase mRNA. Longer exposures of blots, like the one shown in Fig. 3, failed to reveal a reticulocyte band comigrating with the liver species (data not shown).

RNase A Protection Experiments. pG500 and pG900 (see Fig. 1) were used to generate uniformly labeled antisense RNA probes. These two probes, which covered the 5′-terminal and 3′-terminal halves of the coding region of reductase mRNA, respectively, were annealed with total liver, reticulocyte, or yeast RNA and then treated with a mixture of RNases to digest the unhybridized probe. The RNase-resistant products were analyzed on sequencing gels. The results obtained with pG900 are shown in Fig. 4*A*. The probe was protected along its entire length (894 nt) by liver (lane 2) and by reticulocyte (lane 3) RNA. The small difference in length between the protected fragment and the probe not exposed to RNase digestion (lane 1) is due to the removal of extraneous RNA at the extremities of the probe generated by transcription of a portion of pGEM-3 polylinker. Yeast RNA, used as control, was incapable of protecting the probe (lane 4).

These results indicated that the portion of reductase mRNA encoding amino acids 140–300 as well as the first 422 nt of the 3′-noncoding region is the same in reticulocytes and liver. A different result was obtained with the probe generated from pG500 (Fig. 4*B*). As in the case of pG900, this probe was protected along its entire length (454 nt) by liver (lane 2) and not protected by yeast RNA (lane 4). Instead, when reticulocyte RNA was used, in addition to the protected probe of 454 nt a band of ≈390 nt appeared (lane 3), suggesting that reticulocytes contain two reductase mRNAs, one with a coding sequence identical to that of liver and the other differing from the liver transcript. Doubling the RNase

```
1   TAGAACGGTG CCACCACTGT CTTCTTCGCC ACC ATG GGG GCC CAG CTG AGC ACG TTG AGC CGA GTG GTA CTC TCC CCG GTC TGG TTC GTC TAC
r                                    MET Gly Ala Gln Leu Ser Thr Leu Ser Arg Val Val Leu Ser Pro Val Trp Phe Val Tyr
s                                        GLY  -   -   -   -   -   -  Gly His  -   -   -   -   -  Leu  -   -  Leu  -
h                                                                   LEU Gly His Met  -   - Phe  -   -   -   -  Leu  -
94  AGC CTC TTC ATG AAG CTG TTT CAG CGC TCC TCA CCG GCC ATC ACC CTC GAG AAC CCC GAC ATC AAG TAC CCT CTG CGG CTC ATC GAC
r   Ser Leu Phe Met Lys Leu Phe Gln Arg Ser Ser Pro Ala Ile Thr Leu Glu Asn Pro Asp Ile Lys Tyr Pro Leu Arg Leu Ile Asp
s    -   -  Ile  -   -   -   -   -   -   -  Thr  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
h    -   -  Leu  -   -   -   -   -   -   -  Thr  -   -   -   -   -   -  Ser  -   -   -   -   -   -   -   -   -   -
181 AAG GAG ATT ATC AGC CAT GAC ACT CGG CGC TTC CGA TTT GCA CTC CCT TCG CCC CAG CAC ATC CTG GGC CTT CCT ATC GGC CAG CAC
r   Lys Glu Ile Ile Ser His Asp Thr Arg Arg Phe Arg Phe Ala Leu Pro Ser Pro Gln His Ile Leu Gly Leu Pro Ile Gly Gln His
s    -   -  Val  -   -   -   -   -   -   -   -   -   -   -   -   -   -  Glu  -   -   -   -   -   -  Val  -   -   -
h   Arg  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Val  -   -   -
268 ATC TAC CTC TCC ACC AGG ATC GAT GGC AAC TTG GTC ATT CGT CCC TAC ACC CCT GTG TCT AGT GAT GAT GAC AAG GGC CTT GTG GAC
r   Ile Tyr Leu Ser Thr Arg Ile Asp Gly Asn Leu Val Ile Arg Pro Tyr Thr Pro Val Ser Ser Asp Asp Asp Lys Gly Leu Val Asp
s    -   -   - Ala  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Phe  -   -
h    -   -   - Ala  -   -   -   -   -   -   - Val  -   -   -   -   -   - Ile  -   -   -   -   -   -  Phe  -   -
355 TTG GTG GTC AAG GTT TAC TTC AAG GAC ACG CAT CCC AAG TTT CCA GCT GGA GGG AAA ATG TCT CAG TAC CTG GAA AAC ATG AAT ATT
r   Leu Val Val Lys Val Tyr Phe Lys Asp Thr His Pro Lys Phe Pro Ala Gly Gly Lys Met Ser Gln Tyr Leu Glu Asn Met Asn Ile
s    -   -  Ile  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Ser  -  Gly  -
h    -   -  Ile  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Ser  -  Gln  -
442 GGA GAC ACC ATT GAA TTC CGG GGC CCC AAT GGG CTA CTG GTC TAC CAG GGC AAA GGG AAG TTC GCC ATC CGT GCA GAC AAG AAG TCC
r   Gly Asp Thr Ile Glu Phe Arg Gly Pro Asn Gly Leu Leu Val Tyr Gln Gly Lys Gly Lys Phe Ala Ile Arg Ala Asp Lys Lys Ser
s    -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   - Pro  -  Ser  -   -
h    -   -   -   -   -   -   -   - Ser  -   -   -   -   -   -   -   -   -   -   -   -   -   -   - Pro  -   -   -   -
529 AAC CCT GTT GTC AGG ACG GTG AAG TCT GTA GGC ATG ATT GCA GGA GGG ACA GGC ATC ACC CCA ATG CTG CAG GTG ATC CGA GCC GTC
r   Asn Pro Val Val Arg Thr Val Lys Ser Val Gly Met Ile Ala Gly Gly Thr Gly Ile Thr Pro Met Leu Gln Val Ile Arg Ala Val
s   Asp  -   - Ile Lys  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   - Ile
h    -   -  Ile Ile  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
616 TTG AAG GAC CCG AAC GAC CAC ACT GTG TGC TAT CTG CTC TTC GCC AAC CAG TCC GAG AAA GAC ATC CTG CTG CGG CCT GAG CTG GAG
r   Leu Lys Asp Pro Asn Asp His Thr Val Cys Tyr Leu Leu Phe Ala Asn Gln Ser Glu Lys Asp Ile Leu Leu Arg Pro Glu Leu Glu
s   Met  -   -   - Asp  -   -   -   -   - His  -   -   -   -   -   - Thr  -   -   -   -   -   -   -   -   -   -   -
h   Met  -   -   - Asp  -   -   -   -   - His  -   -   -   -   -   - Thr  -   -   -   -   -   -   -   -   -   -   -
703 GAA CTG AGG AAC GAA CAT TCT TCT CGC TTC AAG CTC TGG TAC ACA GTG GAC AAA GCC CCC GAT GCC TGG GAC TAT AGC CAA GGC TTC
r   Glu Leu Arg Asn Glu His Ser Ser Arg Phe Lys Leu Trp Tyr Thr Val Asp Lys Ala Pro Asp Ala Trp Asp Tyr Ser Gln Gly Phe
s    -   -   - Asp  -   -   - Ala  -   -   -   -   -   -   -   -   -   -   -  Glu  -   -   -   -   -   -   -   -   -
h    -   -   - Lys  -   - Ala  -   -   -   -   -   - Leu  -  Arg  -   - Glu  -   -   - Gly  -   -   -
790 GTG AAT GAG GAG ATG ATC AGG GAC CAT CTT CCA CCT CCT GGG GAG GAG ACA CTG ATA CTG ATG TGT GGA CCC CCA CCG ATG ATC CAG
r   Val Asn Glu Glu Met Ile Arg Asp His Leu Pro Pro Pro Gly Glu Glu Thr Leu Ile Leu Met Cys Gly Pro Pro Pro Met Ile Gln
s    -   -   -   -   -   -   -   -   -   -   -   -   - Glu  -   - Pro  - Val  -   -   -   -   -   -   -   -   -   -
h    -   -   -   -   -   -   -   -   -   -   -   -   - Glu  -   - Pro  - Val  -   -   -   -   -   -   -   -   -   -
877 TTT GCC TGT TTG CCA AAC CTG GAG CGT GTG GGC CAT CCC AAG GAG CGA TGC TTC ACC TTC TGATGGCTGG ATGCTGGCCA CTCCCATGCC
r   Phe Ala Cys Leu Pro Asn Leu Glu Arg Val Gly His Pro Lys Glu Arg Cys Phe Thr Phe
s   Tyr  -   -   -   -   - Asp  -   -   -   -   -   -   -   - Ala  -
h   Tyr  -   -   -   -   - Asp His  -   -   -   - Thr  -   -   - Val  -
967  TGCTGCTCAC GCACTCACCA CAACCACCTT CCACCCCTTC CTTCCCCACT ACTGTCCTTT ACCCTGACAT ATGCCCACAT CCATGCTGGG GCCTGGGTTC
1067 AGCCTGGCCT GCCCAGCCCT GGTCATCCAG CTGTACTGGC CCCTGAGGGG CCCCTTTGGG AGCAGGCCTG TGTATCAGGT GGCTTCTGTT GACCACTTTC
1167 TGAATAGGCT TCTGTCTGGT ACTAACTGGC CATTACCAGA GATGGTCCAT GACCACCCCT TTATACACAC ACACATACAC ATACAGAGAC AGAGAGACAG
1267 AGAGACAGAG ATAGACAGAG ATAGACAGAG AGACAGATAC AGACATAGAG ACAGAGAGAG AAGAGAGAGA GAGGAGAGAG AG
```

FIG. 2. Nucleotide sequence of the second clone and deduced amino acid sequence of rat liver cytochrome b_5 reductase. The ≈500-bp fragment was sequenced in pGEM-3, by using the T7 and SP6 promoter primers (Promega Biotec). The ≈900-bp fragment was sequenced after subcloning appropriate overlapping restriction fragments into M13. Numbers on the left refer to nucleotide positions. Nucleotides contributed by *Eco*RI linkers, present at the extremities of the clone, are not included in this figure. The deduced amino acid sequence of the rat protein is shown on line r. The positions at which the bovine liver (line s, ref. 6) and human liver (line h, ref. 8) reductase differ from the rat protein are shown. Positions of identity are indicated by the dashes. The first amino acid of each sequence is indicated in capital letters.

A concentration did not alter the ratio between these two protected fragments (data not shown).

To determine at which extremity of the probe the mismatch with reticulocyte reductase mRNA was localized, an antisense probe lacking 139 nt at the 5′ end (*Ava* I-digested pG500; see Fig. 1) was hybridized to reticulocyte RNA. As shown in Fig. 4*C*, lane 3, after RNase digestion, only one fragment was obtained, corresponding to the entire length of the probe (315 nt). Thus, the mismatch between the liver pG500 probe and the second reticulocyte mRNA species is localized at the 5′ extremity of the probe close to nt 64—i.e., within the coding region ≈30 nt from the start codon.

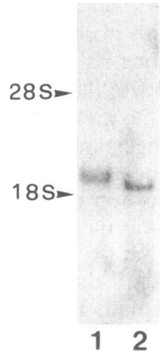

FIG. 3. RNA gel blot of liver and reticulocyte RNA hybridized with reductase probe. RNA molecules were separated on 1% agarose gels containing 2.2 M formaldehyde and Mops buffer (28). Lanes: 1, 1 μg of poly(A)$^+$ liver RNA; 2, 8.5 μg of total reticulocyte RNA. The blot was probed with the *Eco*RI–*Pvu* II fragment of the first clone (see Fig. 1). Hybridization, in 0.75 M NaCl plus 50% (vol/vol) formamide at 42°C, and washing, in 0.015 M NaCl at 60°C, were under high-stringency conditions. 28S and 18S indicate the positions of rRNA markers.

In all these experiments, controls were carried out with sense, instead of antisense, probes. As expected, no protection was obtained (data not shown).

Southern Blot Analysis. The ≈500- and ≈900-nt fragments were used as probes in Southern blots of rat genomic DNA, digested with *Eco*RI or *Bam*HI (Fig. 5). Since the two probes are generated by cleavage with *Eco*RI at a site within the reductase coding sequence, they were expected to recognize different fragments of genomic DNA digested by *Eco*RI. In fact, the ≈500-nt probe recognized a band of 6400 bp (Fig. 5*A*, lane 1), whereas the ≈900-nt probe hybridized to a band of 950 bp (Fig. 5*B*, lane 1). In contrast, both probes recognized the same 14,000-bp band after *Bam*HI digestion of genomic DNA (lanes 2 of *A* and *B*). These results strengthen the hypothesis that a single gene codes for the reductase.

DISCUSSION

In this study, we have isolated a cDNA clone containing the entire coding sequence of rat liver cytochrome b_5 reductase that we have used as probe to study the transcripts encoding the reductase in liver and in reticulocytes. A cDNA encoding human reductase has been isolated by others (8); however,

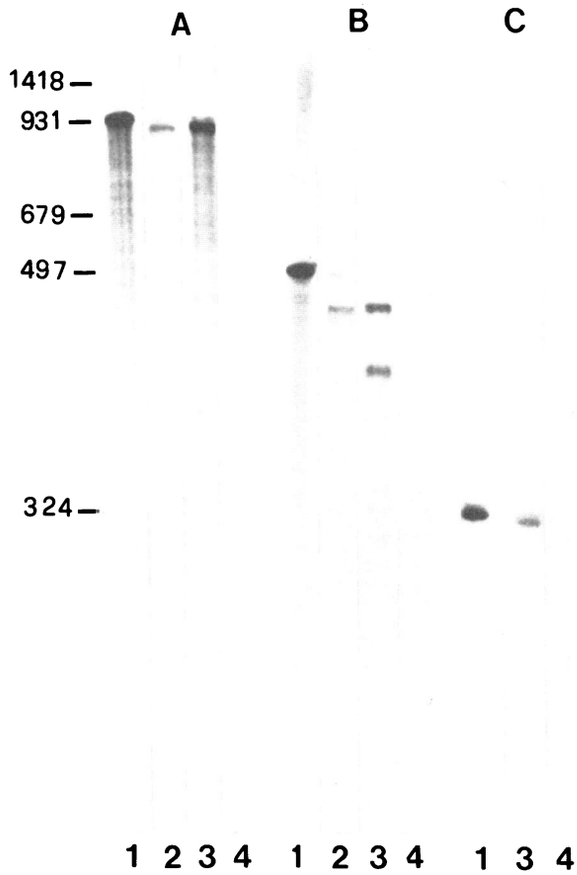

FIG. 4. Analysis of liver and reticulocyte reductase mRNA by RNase mapping. Total liver RNA (6 μg) (lanes 2), 12 μg of total reticulocyte RNA (lanes 3), or 12 μg of total yeast RNA (lanes 4) were hybridized to 0.3×10^6 cpm ($\approx$0.3 ng) of pG900 antisense RNA probe (*A*), pG500 antisense RNA probe (*B*), or a 324-nt antisense RNA probe transcribed from pG500 linearized by *Ava* I digestion (*C*). The samples, in a final volume of 330 μl, were then digested for 15 min at 30°C with RNase A (36 μg/ml) plus RNase T1 (0.084 unit/μg of RNA) in *A* and *B* and with RNase A (72 μg/ml) plus RNase T1 (0.084 unit/μg of RNA) in *C*. The RNase-resistant products were size-fractionated on 6% polyacrylamide/urea sequencing gels and detected by autoradiography with intensifying screens. Numbers on the left indicate positions and length (in nt) of RNA markers synthesized from Gemini-positive control template and from truncated pG500 and pG900 plasmids. Undigested probes are shown in lanes 1.

this clone lacked the extreme 5′ terminus of the coding region.

The sequence analysis of the cloned cDNA confirmed that the reductase is a highly conserved protein (8) and, in addition, showed that the primary translation product differs at its N terminus from the mature protein [sequenced in bovine liver (6)] only by the presence of the initiator methionine. Thus, the reductase is not synthesized as a precursor with an extra peptide destined for removal, as suggested also by results obtained in cell-free translation systems (11, 29).

Cytochrome b_5 reductase is present in three locations: as an integral constituent of endoplasmic reticulum and outer mitochondrial membranes and as a soluble protein in the cytoplasm of erythrocytes. Genetic data in humans (5), as well as the Southern blot analysis presented in the present paper, strongly support the hypothesis that a single gene codes for this enzyme. To pursue our studies on the biogenetic relationships between the reductase in its different locations, we analyzed its transcripts with the RNase A mapping technique (26). This technique seems to be particularly appropriate for picking up small differences between nucleic acid molecules since it can detect even point mutations (26). The results obtained with liver RNA indicate that only one reductase sequence is present in the transcripts of that tissue. If a second liver mRNA coding for mitochondrial reductase did exist, it should have been present in sufficient concentration to be detected in our experiments. In fact, given the known abundance of liver mitochondrial reductase (2) and its rate of synthesis compared to microsomal reductase (10), a hypothetical mRNA coding for the mitochondrial form would represent $\approx$20% of the reductase encoding transcripts of the liver cell. Moreover, if mitochondrial and microsomal reductase are products of the same gene, as suggested by the genetic data (5), differences between the two forms would be expected to be restricted to limited portions of the enzyme. Thus, protected fragments resulting from partial hybridization between the probes and a putative second liver transcript should have been detected. It seems, therefore, reasonable to conclude that only one membrane form of reductase exists in liver and that the same reductase is targeted to both outer mitochondrial and endoplasmic reticulum membranes. This situation is in contrast with that of cytochrome b_5, for which a distinct mitochondrial form has been identified (30). The mechanism of the recognition between the reductase and these two membranes is not yet understood. However, the insertion of the reductase cannot be explained simply by a nonspecific partitioning of its hydrophobic domain into any lipid bilayer, since it is present at low concentrations, or not at all, on all organelles of the hepatocyte except endoplasmic reticulum and outer mitochondrial membranes (2).

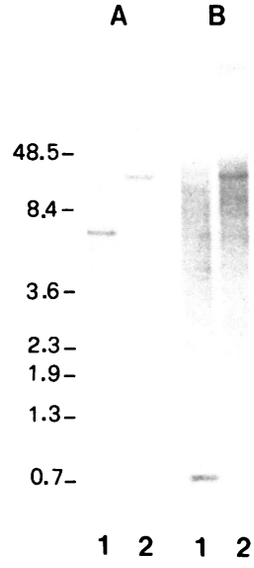

FIG. 5. Southern blot of rat genomic DNA hybridized with reductase probes. DNA (20 μg) was digested with *Eco*RI (lanes 1) or with *Bam*HI (lanes 2) and separated on a 0.7% agarose gel. Hybridization was carried out, as specified in Fig. 3, with the $\approx$500-bp (*A*) or the $\approx$900-bp (*B*) probe. Numbers on the left indicate positions and length in bp ($\times 10^{-3}$) of *Bst*EII-digested and undigested phage DNA markers.

When RNase protection experiments were carried out with reticulocyte RNA, quite a different result from that in liver was obtained. In fact, two transcripts were detected, one of which contained a region of mismatch with the 5′-terminal liver probe. This mismatch was located at nt 60–65 of the probe, $\approx$30 nt downstream of the initiator AUG. We do not know how far to the left of this position the mismatch extended; however, our data indicate that the region of diversity between the second reticulocyte mRNA and the liver probe must be large (>30 nt). In fact, if the difference had been restricted to a few nucleotides, a second protected fragment of $\approx$60 nt would have been generated by RNase

digestion. However, in gels designed to detect small molecules (≥30 nt), no additional protected fragments were observed, indicating that the region of the probe upstream to positions 60–65 had been extensively digested (results not shown).

Since the difference between the soluble and membrane form of the reductase is known to be located at the N terminus (7, 8) and the soluble form is present in erythrocytes and absent in liver, we suggest that the extra transcript present in reticulocytes encodes soluble cytochrome b_5 reductase. This transcript could be generated from the reductase gene by an alternative promoter and/or alternative splicing mechanism. An inconsistency of this hypothesis with previous work is that human soluble reductase has been reported to have as its N-terminal amino acid phenylalanine-26 of the membrane form (7), corresponding to the TTT codon at position 112 of our clone. However, it is possible that soluble reductase is larger than previously thought and may have been partially degraded during purification before sequencing (7), as suggested also by our results on rat soluble reductase (13).

An unexpected finding was that the single band seen on RNA gel blots of reticulocyte RNA appeared to contain two molecules, one completely matched to the liver probes employed. It is, in fact, unlikely that the presence of the larger protected fragment observed in RNase A protection experiments was an artifact due to incomplete digestion of the probe, because the polylinker region of the probe was completely digested and because increasing the concentration of RNase A did not alter the ratio between the two protected fragments. Thus, two transcripts, very similar in size, were probably responsible for the generation of these two protected fragments. Since the reticulocyte "liver-type" transcript completely protected the liver probes, its smaller size compared to that of its counterpart in liver (seen on RNA gel blots) must be explained by the presence of a difference outside of the sequence covered by the probes, possibly at the 3′ terminus of the transcripts.

The possibility should also be considered that the liver-type RNA present in reticulocyte RNA preparations was contributed by the small amount of contaminating leukocytes in our erythrocyte preparations; however, this would imply an enormously high concentration of reductase mRNA in leukocytes (>1000 times higher than in liver), a situation difficult to reconcile with the reported levels of reductase protein in leukocytes (5, 13). It remains surprising that reticulocytes contain so much liver-type transcript, since >90% of reticulocyte reductase is soluble (13). The liver-type transcript, coding for the membrane form, may represent a remnant from more immature erythrocytes. Indeed, membrane-bound reductase has been shown to be present in Friend erythroleukemia cells (31). In any case, the presence in reticulocytes of a reductase-encoding transcript that differs from the liver mRNA in the extreme 5′-terminal portion of the coding sequence supports the idea that soluble reductase is translated from a tissue-specific mRNA.

We thank Drs. M. Mueckler and G. Cairo for their kind gifts of a rat liver cDNA library in λgt11 and a preparation of rat genomic DNA, respectively. We thank Mr. P. Tinelli, Mr. F. Crippa, and Mr. C. Sala for technical collaboration in the preparation of the illustrations; Drs. D. Fesce, G. Fumagalli, and C. Lafortuna for computer advice; Dr. L. Mori for helpful advice on Southern blotting; and Dr. F. Clementi for reading the manuscript. This work was partially supported by Consiglio Nazionale delle Ricerche, Progetto Finalizzato "Ingegneria genetica e basi molecolari delle malattie ereditarie."

1. Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32,** 415–438.
2. Borgese, N. & Pietrini, G. (1986) *Biochem. J.* **239,** 393–403.
3. Tamura, M., Yubisui, T., Takeshita, M., Kawabata, S., Miyata, T. & Iwanaga, S. (1987) *J. Biochem. (Tokyo)* **101,** 1147–1159.
4. Hultquist, D. E., Slaughter, S. R., Douglas, R. H., Sannes, L. J. & Sagavian, G. G. (1978) *Prog. Clin. Biol. Res.* **21,** 199–211.
5. Leroux, A., Junien, C., Kaplan, J.-C. & Bamberger, J. (1975) *Nature (London)* **258,** 619–620.
6. Ozols, J., Korza, G., Heinemann, F. S., Hediger, M. A. & Strittmatter, P. (1985) *J. Biol. Chem.* **260,** 11953–11961.
7. Yubisui, T., Miyata, T., Iwanaga, S., Tamura, M. & Takeshita, M. (1986) *J. Biochem. (Tokyo)* **99,** 407–422.
8. Yubisui, T., Naitoh, Y., Zenno, S., Tamura, M., Takeshita, M. & Sakaki, Y. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3609–3613.
9. Borgese, N. & Gaetani, S. (1980) *FEBS Lett.* **112,** 216–220.
10. Borgese, N., Pietrini, G. & Meldolesi, J. (1980) *J. Cell Biol.* **86,** 38–45.
11. Borgese, N. & Gaetani, S. (1983) *EMBO J.* **2,** 1263–1269.
12. Meldolesi, J., Corte, G., Pietrini, G. & Borgese, N. (1980) *J. Cell Biol.* **85,** 516–526.
13. Borgese, N., Macconi, D., Parola, L. & Pietrini, G. (1982) *J. Biol. Chem.* **257,** 13854–13861.
14. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
15. Davis, R. W., Botstein, D. & Roth, J. R. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
16. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
17. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
19. Werner, D., Chemla, Y. & Herzberg, M. (1984) *Anal. Biochem.* **141,** 329–336.
20. Mueckler, H. & Pitot, H. C. (1985) *J. Biol. Chem.* **260,** 12993–12997.
21. Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning*, ed. Glover, D. M. (IRL, Oxford), Vol. 1, pp. 49–78.
22. Beutler, E., West, C. & Blume, K.-G. (1976) *J. Lab. Clin. Med.* **88,** 328–333.
23. Borgese, N., Pietrini, G. & Gaetani, S. (1987) *J. Clin. Invest.* **80,** 1296–1302.
24. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12,** 7035–7056.
25. Green, M. R., Maniatis, T. & Melton, D. A. (1983) *Cell* **32,** 681–694.
26. Zinn, K., Di Maio, D. & Maniatis, T. (1983) *Cell* **34,** 865–879.
27. Kozak, M. (1983) *Microbiol. Rev.* **47,** 1–45.
28. Seed, B. (1982) in *Genetic Engineering*, eds. Setlow, J. & Hollander, A. (Academic, New York), Vol. 4, pp. 91–102.
29. Okada, Y., Frey, A. B., Guenthner, T. M., Oesch, F., Sabatini, D. D. & Kreibich, G. (1982) *Eur. J. Biochem.* **122,** 393–402.
30. Lederer, F., Ghrir, R., Guiard, B., Cortial, S. & Ito, A. (1983) *Eur. J. Biochem.* **132,** 95–102.
31. Slaughter, S. R. & Hultquist, D. E. (1979) *J. Cell Biol.* **83,** 231–239.