

A Single mRNA, Transcribed from an Alternative, Erythroid-specific, Promoter, Codes for Two Non-myristylated Forms of NADH-Cytochrome b_5 Reductase

Grazia Pietrini,* Diego Aggujaro,* Paola Carrera,* Jan Malyszko,‡ Alessandro Vitale,§ and Nica Borgese*

*C.N.R. Center for Cytopharmacology and Department of Pharmacology, University of Milan, Italy; ‡Biotechnology Department, Farmitalia-Carlo Erba, Milan, Italy; §C.N.R. Istituto Biosintesi Vegetali, Milano, Italy

Abstract. Two forms of NADH-cytochrome b_5 reductase are produced from one gene: a myristylated membrane-bound enzyme, expressed in all tissues, and a soluble, erythrocyte-specific, isoform. The two forms are identical in a large cytoplasmic domain ($M_r \approx 30,000$) and differ at the NH_2 -terminus, which, in the membrane form, is responsible for binding to the bilayer, and which contains the myristylation consensus sequence and an additional 14 uncharged amino acids. To investigate how the two differently targeted forms of the reductase are produced, we cloned a reductase transcript from reticulocytes, and studied its relationship to the previously cloned liver cDNA. The reticulocyte transcript differs from the liver transcript in the 5' non-coding portion and at the beginning of the coding portion, where the seven codons specifying the myristoylation consensus are replaced by a reticulocyte-specific sequence which codes for 13 non-charged amino acids. Analysis of genomic reductase clones in-

dicated that the ubiquitous transcript is generated from an upstream "housekeeping" type promoter, while the reticulocyte transcript originates from a downstream, erythroid-specific, promoter. In vitro translation of the reticulocyte-specific mRNA generated two products: a minor one originating from the first AUG, and a major one starting from a downstream AUG, as indicated by mutational analysis. Both the AUGs used as initiation codons were in an unfavorable sequence context. The major, lower relative molecular mass product behaved as a soluble protein, while the NH_2 -terminally extended minor product interacted with microsomes in vitro. The generation of soluble reductase from a downstream AUG was confirmed in vivo, in *Xenopus* oocytes. Thus, differently localized products, with respect both to tissues and to subcellular compartments, are generated from the same gene by a combination of transcriptional and translational mechanisms.

BECAUSE short consensus sequences involved in protein targeting and posttranslational modifications are often distinct from domains mediating protein function, it is possible for cells to produce differently localized versions of the same protein. For example, the consensus sequence which determines the covalent attachment of myristic acid consists of only six amino-terminal amino acids (Towler et al., 1988; Kaplan et al., 1988; Buss et al., 1988), and this co-translational modification plays a role in protein targeting (Buss et al., 1986; Resh and Ling, 1990). Thus it should be possible to produce myristylated and non-myristylated versions of the same protein, with different intracellular destinations. To investigate mechanisms by which the cell might achieve this goal, we have studied the biogenetic relationship between the two forms of the flavoprotein NADH-cytochrome b_5 reductase (reductase), which are known to be produced from one gene (Leroux et al., 1975; Kobayashi

et al., 1990), and which are: (1) a myristylated, membrane-associated enzyme, involved in fatty acid desaturation (Strittmatter et al., 1974), and present on ER and on outer mitochondrial membranes of liver (Borgese and Pietrini, 1986) and of other tissues (Tamura et al., 1987); and (2) a soluble erythroid form, once known as methemoglobin reductase (Hultquist and Passon, 1971).

The membrane form of the reductase has a molecular weight of 34,000 and consists of a hydrophilic, cytoplasmically exposed domain (flavo-peptide domain) and an NH_2 -terminal membrane anchor (Kensil and Strittmatter, 1986). Membrane-bound reductase is N-myristylated (Ozols et al., 1984), both in its outer mitochondrial membrane and its ER locations (Borgese and Longhi, 1990). Although the myristic acid may contribute to targeting and to the overall hydrophobicity of the membrane anchor, it is probably not sufficient for the interaction of reductase with membranes (Gordon et al., 1991). The NH_2 -terminal domain of the reductase contains a stretch of 14 non-charged amino acids (residues 11–24 of the primary translation product), and

Paolo Carrera's present address is Scientific Institute "S. Raffaele", Milan, Italy.

studies with the isolated NH₂-terminal peptide have shown that this domain penetrates deeply into bilayers (Kensil and Strittmatter, 1986). Consistently, the reductase behaves as an integral membrane protein, since its membrane association is resistant to alkali (Borgese and Pietrini, 1986), and since it binds neutral detergents (Borgese et al., 1982). Thus, the primary structure of the membrane anchor of the reductase can be thought of as consisting of two parts: an initial stretch of amino acids (from position 1 to position 7) which contributes the consensus sequence for myristylation (Towler et al., 1988), and a downstream stretch of hydrophobic residues, which interact with the lipid bilayer. The precise topology of this domain within the membrane is not known.

The soluble, erythrocyte-specific form of the reductase, plays an important role in methemoglobin reduction (Hultquist and Passon, 1971). The primary structure of this protein, purified from human erythrocytes, is identical to that of the cytoplasmic domain of the membrane form, with the first amino acid corresponding to Phe-26 of the membrane enzyme (Yubisui et al., 1986, 1987). This observation has led to the suggestion that the soluble form might be generated from the membrane-bound enzyme by proteolytic processing during erythrocyte maturation (Yubisui et al., 1987). In contrast, on the basis of evidence generated in our laboratory, which indicated the presence in erythrocyte precursors (reticulocytes) of a second transcript for the reductase, we proposed that soluble reductase might be generated by an alternative promoter or alternative splicing mechanism (Pietrini et al., 1988). The difference between the reticulocyte (R)¹ and liver (L) transcripts was mapped to the extreme 5' terminus of the coding region (Pietrini et al., 1988), suggesting that the R transcript might lack only the codons specifying the myristylation consensus, and thus encode a nonmyristylated version of the reductase.

In the present study, we show that, indeed, an exon encoding the myristylation consensus is excluded from the R-mRNA, which is transcribed from a downstream, tissue-specific promoter, and, in addition, that the R-transcript generates the soluble reductase by an unusual mechanism of alternative initiation of translation.

Materials and Methods

General

Most of the work involving nucleic acids was carried out by standard procedures, described in laboratory manuals ("Current Protocols in Molecular Biology" (John Wiley and Sons, New York) and Sambrook et al. (1989)). For Southern blotting, Hybond N+ filters (Amersham International, Amersham, UK) were used. Blots were hybridized with ³²P-labeled DNA fragments or oligonucleotides. DNA fragments were labeled by random priming, and oligonucleotides were 5' end labeled using T₄ polynucleotide kinase. Sequencing of recombinant plasmids or of fragments subcloned into M13 vectors was carried out with Sequenase (United States Biochemicals, Cleveland, OH), following the instructions of the manufacturer. RNA was prepared from Sprague-Dawley rat tissues or reticulocytes by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Reticulocytes were obtained from phenylhydrazine-treated rats as previously described (Pietrini et al., 1988). For the S1 nuclease protection experiments, polyA⁺ RNA was selected from total RNA by oligo(dT)-cellulose affinity chromatography. 1st strand cDNA, to be used in polymerase chain reaction (PCR) experiments, was synthesized with se-

quence-specific oligonucleotide primers and total RNA. Reagents were from Amersham International, and the reactions were carried out according to the instructions of the manufacturer. PCR was carried out in a thermocycler from Perkin-Elmer Cetus (Norwalk, CT) or in an RNA-DNA amplifier from Violet (Biostar, Milan, Italy). *Thermus aquaticus* DNA polymerase and reaction buffer were from Perkin-Elmer Cetus or from Promega Biotec (Madison, WI). DMSO was added to all samples at a final concentration of 10%. SDS-PAGE of [³⁵S]methionine-labeled translation products was carried out on reduced, alkylated samples, as previously described (Borgese et al., 1982).

Oligonucleotides

Oligonucleotides were synthesized with a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA). Crude oligonucleotides were desalted by gel filtration, or purified on polyacrylamide sequencing gels. The sequences of the oligonucleotides, and the experiments in which they were used, are listed in Table I. In the text, oligonucleotides are referred to with the Roman numerals used in the Table.

Cloning of Reticulocyte Reductase cDNA with Anchored PCR

Reticulocyte (R) 1st strand reductase cDNA was obtained from 0.5 μg of reticulocyte total RNA and a sequence-specific primer derived from the sequence of the liver (L) clone (oligo I; see also Fig. 2). The cDNA was purified, tailed with dATP, and amplified with an internal 3' primer (oligo II), and two 5' primers (oligo III—"anchor-primer-" and oligo IV—"anchor-"), according to the RACE protocol of Frohman et al. (1988). After 41 cycles, the presence of amplified reductase cDNA was verified by Southern blotting with a cDNA probe containing the first 140 bp of liver reductase cDNA. One-fifth of the sample was then reamplified as above, but with omission of the 5' anchor-primer. The re-amplified cDNA was cut with Xho I at an internal site and within the 5' anchor (see Fig. 2) and ligated into pGem7Zf+ (Promega Biotec). Transformants were screened for reductase clones by colony hybridization with the 140-bp Xho I fragment of L-cDNA (see Fig. 2).

S1 Nuclease Protection

Single-stranded anti-sense probes were generated by elongation of ³²P end-labeled oligonucleotide primers (specific radioactivity: 0.5–1.0 × 10⁷ dpm/pmol) with Klenow DNA polymerase, on templates provided by reductase genomic fragments, subcloned into M13 vectors in the sense orientation. A Sau 3a fragment, covering exon 1, was primed with oligo V, and a Nco I fragment, covering exon 2, was primed with oligo VI (see Fig. 3). After elongation, the probes were cut at the Eco RI site of the M13 poly-linker, and purified on denaturing gels.

10 fmol of single-stranded probe were hybridized overnight to 7 μg of poly(A⁺) RNA from rat liver or reticulocytes or of yeast total RNA in 30 μl of buffer containing 40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide. Hybridization was at 48°C for the exon 1 probe versus liver or yeast RNA (see Fig. 3 D) and at 45°C for the exon 2 probe versus reticulocyte or yeast RNA (see Fig. 3 E). After hybridization, the samples were diluted with 10 volumes of digestion buffer (0.28 M NaCl, 0.05 M Na⁺ acetate, pH 4.5 mM ZnHSO₄, 10 μg/ml yeast tRNA), and incubated with S1 nuclease for 1 h. The concentration of S1 nuclease and temperature of incubation were 2,000 U/ml and 40°C, respectively, for the samples hybridized to the exon 1 probe (see Fig. 3 D); 500 U/ml and 30°C, respectively, for the samples hybridized to the exon 2 probe (see Fig. 3 E). Samples were analyzed on 6% polyacrylamide-urea sequencing gels.

Construction of Plasmids for Transcription-translation Experiments

cDNAs were subcloned into pGEM-3 (Promega Biotec) in an orientation which yielded synthetic mRNAs by transcription from the SP6 promoter. The identity of all constructs was confirmed by sequencing.

Details of the constructs are shown in Fig. 1. L-cDNA had originally been subcloned as two separate fragments (pG500 and pG900) because of an internal Eco RI site in the reductase sequence (Pietrini et al., 1988). A plasmid containing the entire coding sequence for the membrane form of the reductase (L-mRNA) was obtained from plasmids pG500 and pG900. pG500 (Fig. 1, line J), which contains the 5' 460 bp of L-cDNA, was restricted at the unique Nco I site (position 34). The 3' recessed end was

1. *Abbreviations used in this paper:* L, liver; PA, polyacrylamide; PCR, polymerase chain reaction; R, reticulocyte.

Table I. Oligonucleotides Used in the Present Study

Oligo	Sequence	Position in cDNA clones or other specifications		Use
		cDNA	Position [‡]	
I	CCGGAATTC AATGGTGT	L-cDNA [§]	462 to 446	primer for reverse transcriptase (Fig. 2)
II	ATTCATGTTTCCAGGT	L-cDNA [§]	438 to 422	3' primer for anchored PCR (Fig. 2)
III	GACTCGAGTCGACATCGA(T) ₁₇	—	—	5' primer for anchored PCR—early cycles (Fig. 2)
IV	GACTCGAGTCGACATCGA	—	—	5' primer for anchored PCR—late cycles (Fig. 2)
V	TGCTCAGCTGGGCCCCCAT	L-cDNA [§]	52 to 34	Generation of single-stranded probe for S1 protection (Fig. 3)
VI	AACAGGCAGAGAGGCAGTC-CAGAGC	R-cDNA	35 to 11	Probe for Southern blot and generation of single-stranded probe for S1 protection (Fig. 3)
VII	CTTGATGTCGGGGTTCGAG	L-cDNA [§]	159 to 139	3' primer for amplification in expression studies (Fig. 4) and in construction of plasmid pGbk 9, pGbk 11, and pGbk 13 (Fig. 1)
VIII	TGTAGAACGGTGCCACCACTG	Exon 1**	−35 to −14	5' primer for amplification (Fig. 4)
IX	GCACTTGAGCCTGCTGTCCAT	Exon 2 ^{‡‡}	−35 to −14	5' primer for amplification (Fig. 4)
X	CATGAAGAGGCTGTAGACGAA-CCAGACCGG	L-cDNA [§]	105 to 76	Probe for Southern Blot (Figs. 3 and 4)
XI	AAAGCTTGAGCCTGCTGTCCA-TCT	R-cDNA	−32 to −13 ^{§§}	5' primer for amplification of R-cDNA: construction of pGbk9, pGbk10, pGbk14 (Fig. 1)
XII	TCTCGAGGGTGATGGCCGGTG-AGGAGCGCTGAAACAGCTTGA-TGA	L-cDNA [§]	145 to 101	3' mutant primer for amplification of R-cDNA: construction of pGbk 10 (Fig. 1)
XIII	GTTTCTCGAGGGTGATGGCCGGTGAGGAGCGCTGAAACAGCTTGCTGA	L-cDNA [§] R-cDNA	147 to 101	3' mutant primer for amplification of R-cDNA: construction of pGbk14 (Fig. 1)
XIV	GTTGCATGCTTGGCCCGCTGCTCTGGACTGCCTCTCTGCCTGTTTGGAGCCGAGTCGTA	R-cDNA	−5 to 54	5' mutant primer for amplification of R-cDNA: construction of pGbk11 (Fig. 1)
XV	GTTGCATGCTTGGCCCGCTGCTCTGGACTGCCTCTCTGCCTGTTTGGAGCCGAATGGTA		−5 to 54	5' mutant primer for amplification of R-cDNA: construction of pGbk13 (Fig. 1)

* All sequences are given from 5' to 3'.

‡ The numbering refers either to the sequence in Pietrini et al. (1988) for L-cDNA or to sequences given in Figures of the present paper. When the first number is larger than the second one, the sequence of the oligonucleotide is complementary to the indicated region.

§ Pietrini et al. (1988). Downstream to nt. 54, the sequence is common to L- and R-cDNA.

|| See Fig. 1 B.

** See Fig. 2 B.

‡‡ See Fig. 2 C.

§§ The first four bases of this oligonucleotide do not belong to R-cDNA, but provide a Hind 3 site.

||| Mutant bases are in bold letters.

filled, and the cDNA fragment was excised with Eco RI and religated into pGEM 3 restricted with Sma I and Eco RI. The resulting plasmid was then cut with Bam HI and Eco RI (Fig. 1, line 2). pG900 (Fig. 1, line J), which contains the 896 bp of reductase cDNA downstream to the Eco RI site, was cut with Eco RI and at an internal Pvu 2 site in the 3' noncoding region (Fig. 1, line 2). The two fragments, obtained from pG500 and pG900 (line 2), were force cloned into pGEM 3 cut with Bam HI and Sma I, to generate plasmid pGbk2 (Fig. 1, line 3), which contains the entire coding sequence for the myristylated form of the reductase, but lacks the 5' noncoding sequence. To obtain a construct containing also the 5' noncoding portion of L-cDNA, pGbk2 was restricted with Bam HI and Xho 1, and then ligated to the Bam HI-Xho 1 fragment of pG500 (Fig. 1, line 4a) to obtain pGbk 3 (Fig. 1, line 5a). To construct a plasmid containing R-cDNA, the fragment which had been cloned by anchored PCR (Fig. 2), and which contained a 5' T₂₀ tail, was cut at the internal Xho 1 site and at the Sal 1 site of the 5' PCR primer. The resulting fragment was ligated to pGbk2 cut with Sal 1 and Xho 1 (Fig. 1, line 4b), to obtain pGbk4 (line 5b). The T₂₀ tail as well as the 5' non coding sequence were removed from this plasmid by removing the 5' Sph 1 fragment (Fig. 1, line 6a) and religating the plasmid, to obtain pGbk5 (Fig. 1, line 7, left). A similar construct, but with deletion of the 1st ATG of the reductase open reading frame (see Fig. 2 B), was obtained by blunting the 3' protruding end of the cut Sph 1 site (Fig. 1, line 6a, middle), before religating to obtain pGbk6 (Fig. 1, line 7, middle). A plasmid containing the 5' noncoding sequence of R-cDNA and lacking the T₂₀ tail

was obtained as follows: a 159-bp fragment of pGbk4, starting immediately downstream to T₂₀, was amplified with a 3' primer spanning the Xho 1 site (oligo VII, Table I) and a 5' primer with a Hind 3 site added at the 5' extremity (oligo XI, Table I). This fragment was cut with Hind 3 and Xho 1 and ligated to pGbk4 restricted with the same enzymes (Fig. 1, line 6b), to obtain pGbk9 (line 7, right). pGbk9 was used for the construction of mutants in the GTG codon at position 48 or in the 2nd in-frame ATG codon at position 88 (see Fig. 2 B), by replacement of the Sph 1-Xho 1 or Hind 3-Xho 1 fragment, respectively, with mutant fragments generated by PCR. These were obtained either with a mutant 5' primer spanning the Sph 1 site (oligos XIV and XV, Table I), and a non-mutant 3' primer spanning the Xho 1 site (oligo VII, Table I), to generate mutations in the GUG codon (pGbk11 with GUG→GUC and pGbk13 with GUG→AUG), or with a non-mutant 5' primer containing a Hind 3 site (oligo XI, Table I) and mutant 3' primers spanning the Xho 1 site (oligos XII and XIII, Table I), to obtain mutations in the 2nd AUG codon (pGbk10 with AUG→AUC and pGbk14 with AUG→AGC).

In Vitro Transcription and Translation

Linearized plasmids were transcribed SP6 RNA polymerase from Promega Biotec, according to the protocol of the manufacturer for cotranscriptional capping with m⁷(5')Gppp(5')G.

In vitro translations with synthetic mRNAs in the presence of high

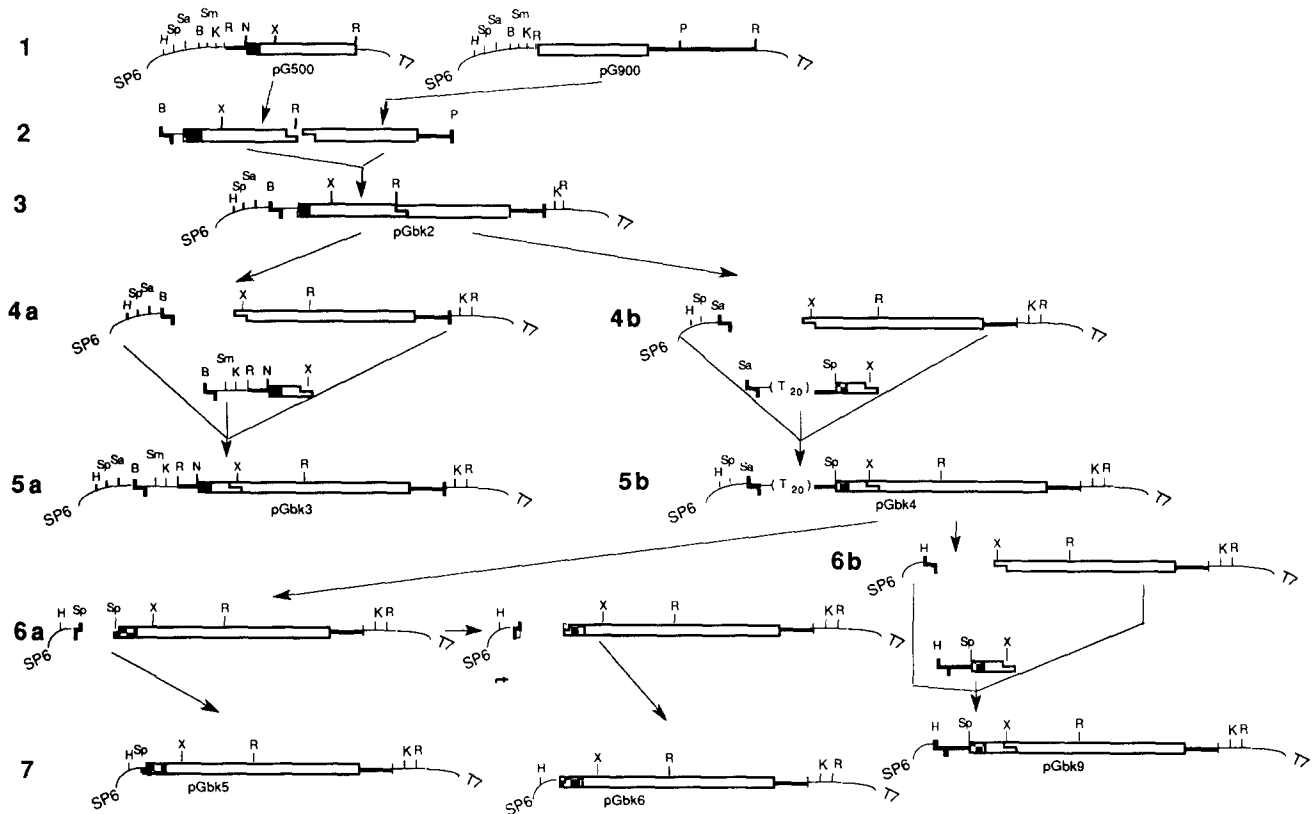


Figure 1. Construction of plasmids for synthesis of wild-type and mutant reductase mRNAs. Open rectangles represent coding region of reductase cDNA common to L and R types, filled and checkered rectangles represent coding sequence specific to L- and R-cDNA, respectively. Thick lines represent noncoding portions of reductase cDNA, thin lines represent pGEM 3 DNA. Abbreviations for restriction sites are: B, Bam HI; H, Hind 3; K, Kpn 1; N, Nco 1; P, Pvu 2; R, Eco RI; Sa, Sal 1; Sm, Sma 1; Sp, Sph 1; X, Xho 1. The indicated sites in the pGEM 3 polylinker are only those which were used for the constructs. See text for further explanations.

specific activity [35 S]methionine were performed in nuclease-treated reticulocyte lysates supplied by Amersham Corp. (Arlington Heights, IL) or Promega Biotec. Lysates were first passed through a Sephadex G-50 column and then supplemented with the components required for protein synthesis at the concentrations previously used (Borgese and Gaetani, 1983). Concentrations of K^+ acetate, Hespes- K^+ , pH 7.4, and Mg^{2+} acetate were 110, 6, and 1.1 mM, respectively.

In Vitro Interaction of Translation Products with Microsomal Membranes

For the posttranslational interaction of the different forms of reductase with microsomes, translation was stopped by incubation of the samples for 10 min at 30°C with 1/4 volume of 8 mM cycloheximide, 8 μ g/ml RNase A in 110 mM KCl, 1.1 mM Mg^{2+} acetate. 8- μ l aliquots of these samples were then supplemented with 6 μ l of a suspension of rat liver microsomes prepared by differential centrifugation (Borgese and Pietrini, 1986) at 8 mg protein/ml in 0.25 M sucrose, 0.1 mM EDTA, 1 mM Tris-HCl, pH 7.4. Controls received only the buffer. After incubation for 30 min at 30°C, the samples were diluted with 36 μ l of 0.01 N NaOH in 0.15 M sucrose and layered over a 150 μ l cushion of 0.4 M sucrose, containing 0.01 N NaOH, in ultracentrifuge tubes of the TLA 100 rotor (Beckman Instruments, Palo Alto, CA). After centrifugation at 65,000 rpm for 30 min at 4°C in the TL-100 ultracentrifuge, the supernatants (cushion + load zone) were collected and precipitated with TCA, while the alkali-resistant pellets were resuspended with 20 μ l of water. Pellets and supernatants were analyzed by SDS-PAGE.

Microinjection and Subfractionation of *Xenopus Laevis* Oocytes

Isolation and microinjection of *Xenopus laevis* oocytes with synthetic, capped mRNAs (deriving from 7.5 ng of plasmid) was carried out as de-

scribed in Ceriotti et al. (1991). 30 min after injection, batches of five oocytes were pulse labeled for 2 h in 5 μ l/oocyte of modified Barth's medium (Ceriotti et al., 1991) supplemented with high specific activity [35 S]methionine (1 μ Ci/ μ l). The oocytes were extensively washed, homogenized, and fractionated into membrane and supernatant fractions as described for "Method B" by Rapoport (1981). Buffers were supplemented with protease inhibitors as follows: 1 mM PMSF, 0.010 T.I.U./ml aprotinin, antipain, chymostatin, leupeptin, pepstatin, and bestatin, all at 2 μ g/ml.

For immunoprecipitation, equivalent aliquots of the membrane and supernatant fractions were adjusted to contain: a final concentration of monovalent ions (K^+ + Na^+) of 150 mM; a final concentration of ethylenediamine-tetraacetic acid equal to that of the $MgCl_2$ present in the samples plus an additional 5 mM; 40 mM Tris-HCl, pH 7.4, 0.04 mM cold methionine, protease inhibitors as above, 2% Triton-X-100, and 0.25% gelatin. Samples were cleared by centrifugation for 10 min at 40,000 rpm in the TLA-100.3 rotor. Reductase was immunoprecipitated from the supernatants with affinity-purified anti-rat reductase antibodies and protein A-Sepharose Cl-4B (Pharmacia, Uppsala, Sweden), as previously described (Borgese and Gaetani, 1980), but gelatin was included in the wash buffer.

Results

Cloning of Reticulocyte Reductase cDNA

We previously showed, with RNase A protection experiments, that rat reticulocytes contain a second transcript for reductase, differing from the L (liver) mRNA within the first 50–60 nts, and suggested that this second transcript might encode the soluble form of the reductase (Pietrini et al., 1988). To clone this R (reticulocyte) transcript, we used an anchored PCR protocol (Frohman et al., 1988), as described

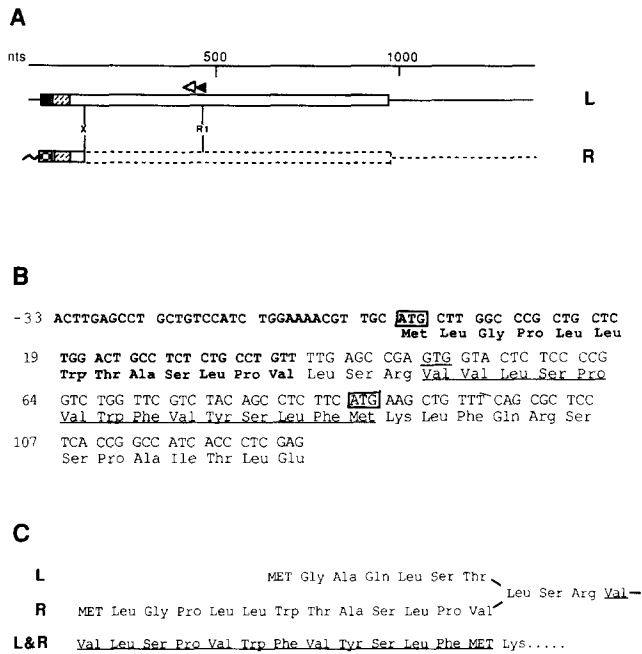


Figure 2. Strategy for cloning the rat reticulocyte reductase cDNA (R-cDNA) and relationship between reticulocyte (R) and liver (L) cDNAs. (A) In L (liver reductase cDNA - sequence in Pietrini et al., 1988), the coding sequence (nts 34–936) is shown as a box and the 5' and 3' noncoding sequences as lines. The filled and hatched areas at the beginning of the coding sequence indicate the first seven codons of the reductase sequence (myristylation consensus) and a subsequent stretch of 14 codons specifying hydrophobic amino acids, respectively. The filled and open arrowheads indicate the positions of the primers used for first strand cDNA synthesis from reticulocyte RNA (*oligo I*, Table I) and for amplification with the RACE protocol (*oligo II*, Table I), respectively. In R, the portion of L-cDNA coding for the myristylation consensus is replaced with a different coding sequence (■). The 5' noncoding sequences are also different (swiggled line in R, versus straight line in L). The region downstream to the Xho 1 site, drawn with dashed lines, was not cloned in the present study, but RNase A protection experiments indicate that it is identical to the L form (Pietrini et al., 1988). Abbreviations for restriction enzymes are: *R*I, Eco RI; *X*, Xho 1. (B) Sequence of 5' Xho 1 fragment of R-cDNA. Portion unique to R-cDNA is bold faced, portion common to R- and L-cDNA is in normal lettering. Two ATG codons in frame with subsequent reductase reading frame are boxed. An in-frame GUG codon is underlined. 14 hydrophobic amino acids, encoded in the common portion of the 2 cDNAs, are underlined. (C) Comparison of predicted NH₂-terminal amino acid sequences of products encoded in L and R-cDNAs. (B) This sequence is available from the EMBL/GenBank under accession number X65191.

in the Materials and Methods section. Transformants were screened for reductase clones with an L-cDNA probe, and three randomly selected positive clones were sequenced: two of these contained an Xho 1 fragment identical to that of L-cDNA, whereas one contained a different sequence upstream of codon 8 of L-cDNA. The presence of L-type mRNA in reticulocytes was not surprising, since it had been detected also by the RNase A protection experiments (Pietrini et al., 1988). The relationship between L- and R-cDNA is illustrated in Fig. 2. The 5' noncoding region as well as the first codons of the 2 cDNAs were different. More specifically, the first ATG of L-cDNA, in the expected con-

sensus for initiation (Kozak, 1989; see Fig. 3 B) is followed by six codons which specify a sequence (Gly-Ala-Gln-Leu-Ser-Thr: Fig. 2 C) conforming to the minimum myristylation consensus (Buss et al., 1988; Towler et al., 1988). Instead, in R-cDNA, the first ATG was not in a favorable context for initiation (Fig. 2 B), and was followed by 12 codons, not present in L-cDNA, in frame with the subsequent reductase reading frame (Fig. 2, B and C). Thus, the consensus for myristylation was not encoded in this transcript. The full-length R-reductase cDNA was not cloned in the present study, but our previous work had indicated that, downstream to the differing region, the two forms of reductase mRNA are the same throughout the coding region and until nt 1,348 of L-cDNA (Pietrini et al., 1988). We have not investigated whether there are differences downstream to this position, however, Northern blotting showed that the transcripts in reticulocytes and in liver were of a similar size (Pietrini et al., 1988).

R-reductase mRNA Is Generated from a Tissue-specific, Downstream Promoter

Since it is known that the soluble and membrane-bound forms of the reductase are generated from one gene (Leroux et al., 1975; Kobayashi et al., 1990), it seemed likely that the two transcripts that we had identified were the products of transcription from two alternative promoters. However, it was also possible that the two mRNAs had a common 5' extremity, not present in our clones, and were generated by an alternative splicing mechanism. The exon-intron organization of the rat reductase gene has been reported (Zenno et al., 1990), however, since the authors did not know of the existence of R-reductase mRNA, they didn't search for the relevant exon. To study the structure of the 5' portion of the reductase gene, a rat liver genomic library in lambda charon 35 (Falany et al., 1987), kindly provided by Drs. Thomas Beck and Charles Kasper (University of Wisconsin, Madison, WI), was screened with cDNA probes specific for L-, R-cDNA, or for regions common to the two cDNAs. Three overlapping clones were isolated, of which one, clone B2,3, recognized by probes specific both for L- and for R-cDNA, was selected for further study (Fig. 3).

As expected, the cDNA sequence unique to the L form, i.e., the 5' noncoding sequence, the initiation codon and the six codons specifying the myristylation consensus, were contained in a separate exon (which we refer to as exon 1), followed by an intron donor site (GT) in agreement with previously reported data for the human (Tomatsu et al., 1989) and rat (Zenno et al., 1990) genes. The region upstream of the 5' extremity of our cDNA clone has features found in promoters of genes lacking tissue-specificity (Dyran, 1986), and has already been described by others (Zenno et al., 1990).

The cDNA sequence unique to the R form (exon 2) was found ≈2,000-bp downstream to exon 1, and was also followed by an intron donor site (Fig. 3 C). The region upstream of our R-cDNA clone has a TATA box between nts -30 and -25 relative to the start of the cDNA clone (underlined between positions -63 and -58 of Fig. 3 C), and two CAAT-like sequences (consensus: GG T/C CAATCT; Benoist et al., 1980) 65- and 115-bp upstream to the start of the cDNA clone (underlined between positions -102 and -97 and between positions -145 and -137 in Fig. 3 C). More interestingly, immediately upstream to the first CAAT-

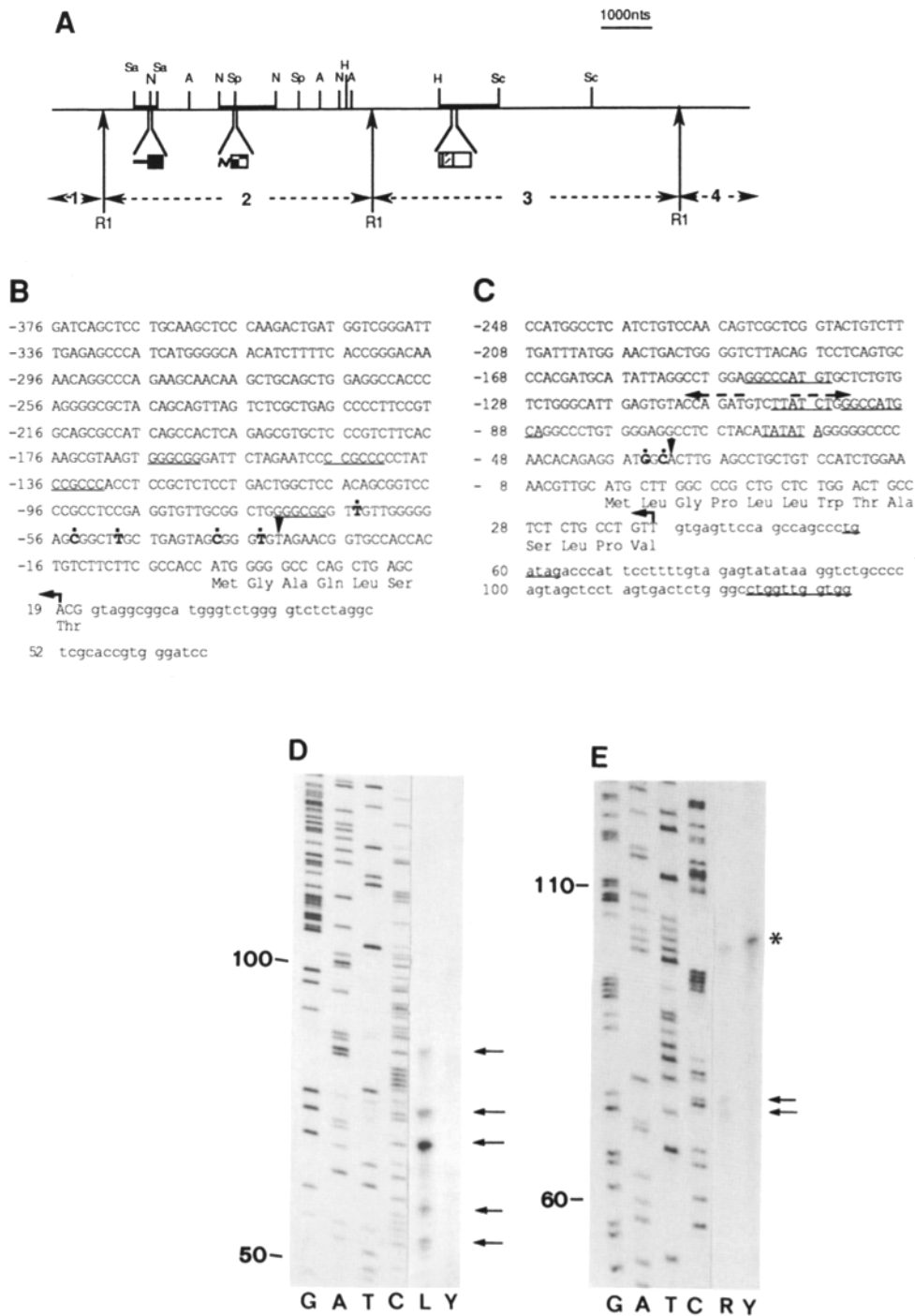


Figure 3. An exon encoding the myristylation consensus is excluded from the reticulocyte transcript by an alternative promoter mechanism. (A) Results of restriction mapping and Southern blot analysis of genomic clone B2.3. Eco R1 digestion of this lambda charon 35 clone yielded four fragments, which were ordered on the basis of hybridization to cDNA or oligonucleotide probes. A 443 bp Sau 3a fragment and a 1,133-bp Nco 1 fragment contained in fragment 2 (**bold lines**), which hybridized to probes specific for L- and R-cDNA, respectively, were sequenced (see B and C). The positions of the L-cDNA (■) and R-cDNA (□) specific sequences within these fragments are shown. The Hind 3-Sac 1 fragment, contained in fragment 3 (**bold**), which was recognized by a probe corresponding to the beginning of the common portion of L- and R-cDNA (*oligo X*, Table I) was also sequenced (sequence not shown in this figure). The position of the first exon common to L and R-cDNA (▣) within this fragment is indicated. Abbreviations for restriction enzymes are: A, Acc 1; H, Hind 3; N, Nco 1; R1, Eco R1; Sa, Sau 3a; Sc, Sac 1; Sp, Sph 1. (B) Sequence of Sau 3a fragment (containing exon 1, expressed in the L transcript). (C) Sequence of the first 375 nts of the Nco 1 fragment (containing exon 2, expressed in the R transcript). The downward pointing arrowheads indicate the start of the L- (B) and R- (C) cDNA clones. For both sequences, nt 1 is the A of the first ATG in frame with subsequent reductase reading frame. Introns are shown in lower-

case lettering. Backward pointing arrows (↵) indicate the start of the single-stranded antisense probes used in the S1 protection experiments shown in D and E, respectively. Upstream of the downward pointing arrowheads, transcriptional initiation sites, determined by S1 protection, are indicated by dots over the bold-faced letters. The sequence in B corresponds to the one published by Zenno et al. (1990), except for two one-base substitutions, at position -351 and -273; four GC boxes are underlined. In C, various possible *cis* regulatory sequences are underlined (see text for details). The dashed arrows pointing in opposite directions indicate an inverted repeat encompassing part of a GATA-1 recognition sequence. (D and E) Determination of transcriptional initiation sites for exon 1 (D) and exon 2 (E) by S1 protection. (D) A ³²P-labeled, single-stranded, antisense probe, covering the region between nts 19 and -376 of the sequence of B, was hybridized to poly A+ RNA from liver (lane L) or to the same amount of total yeast RNA (lane Y). (E) A similar single-stranded probe, covering the region between nts 39 and -248 of the sequence of C, was hybridized to poly A+ RNA from reticulocytes (lane R) or to total yeast RNA (lane Y). After hybridization and nuclease S1 digestion (see Materials and Methods for details), the protected fragments were analyzed on sequencing gels, in parallel with a sequencing ladder (lanes G, A, T, and C), obtained from the same cloned genomic fragments and oligonucleotide primers which were used for the generation of the single-stranded probes. Numbers on the left indicate size (number of nts) of fragments. Arrows point to fragments specifically protected by rat liver (D) or reticulocyte (E) RNA. The asterisk in panel E indicates a non-specific band, obtained also with yeast RNA. (C) This sequence is available from EMBL/GenBank under accession number X65190.

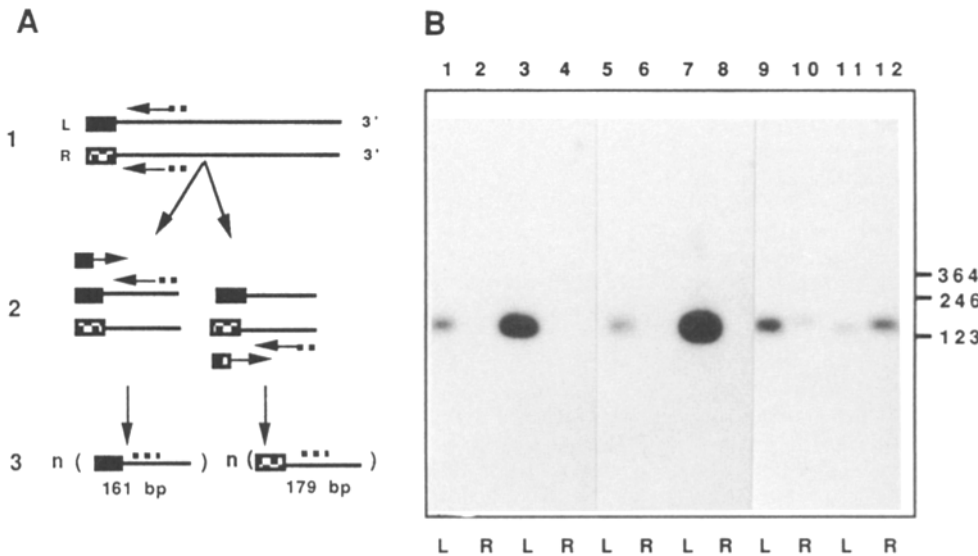


Figure 4. Tissue specificity of the expression of R-reductase mRNA. (A) Design of the experiment. (1) 1.25 μ g of total RNA from different tissues, containing unknown proportions of L-mRNA (5' end marked with closed box) and R-mRNA (5' end marked with checked box) were used as templates for reverse transcription with a primer within the sequence common to the 2 mRNAs (*oligo VII*, Table I). (2) The resulting samples, containing the first strand cDNAs, were divided into two: one half (shown on the left) was amplified with a 5' primer specific for exon 1 (*oligo VIII*, Table I), while the other half (shown on the right) was

amplified with a 5' primer specific for exon 2 (*oligo IX*, Table I). The 3' primer was the same as the one used for cDNA synthesis (*oligo VII*) for both samples. (3) After 15 cycles of amplification, 2/5 of each sample was analyzed by Southern blotting from a 2.5% agarose gel. The blot was hybridized with a fourth, 32 P-labeled, oligonucleotide, complementary to the common portion of the two mRNAs, but different from the one used for amplification (*dotted line*, *oligo X*, Table I). The expected sizes for the amplified fragments are indicated. (B) Result of Southern blot analysis. Each two adjacent lanes, indicated as L and R, show the product of amplification of the same first-strand cDNA with a 5' primer specific for exon 1 or exon 2, respectively. First-strand cDNAs were synthesized from RNA from: lanes 1 and 2, brain; lanes 3 and 4, heart; lanes 5 and 6, kidney; lanes 7 and 8, skeletal muscle; lanes 9 and 10, liver; lanes 11 and 12, reticulocytes. Numbers on the right indicate size (in bp) of standards.

like sequence is a sequence perfectly matching the recognition site for the erythroid transcription factor GATA-1 (previously known as Eryfl, NF-E1, or GF-1; Evans and Felsenfeld, 1989) in inverted orientation (inverted consensus: T/C TATC A/T; sequence underlined between positions -102 and -97 in Fig. 3 C). This transcription factor is highly restricted to the erythroid lineage and appears to play a general role in the regulation of erythroid-specific genes (Pevny et al., 1991). Part of this GATA-1 recognition sequence is contained in an inverted repeat, shown by the dashed arrows of Fig. 3 C. Another GATA-1 binding site, in forward orientation (consensus: A/T GATA A/G), is present at the beginning of the intron (underlined between positions 58 and 63 in Fig. 3 C). Also present at the beginning of the intron is a "GT box" (underlined between position 125 and 133). This sequence (GnnnGGTGG) is often found close to the GATA-1 recognition site in erythroid promoters, and is thought to provide a binding site for a factor which may cooperate with GATA-1 (Philipsen et al., 1990). This GT box overlaps the sequence starting at position 123, which matches the consensus CTGGNTNGNGC (except for the last position) and which provides a binding site for the factor α -CP2 in the murine α -globin promoter (Kim et al., 1990).

A third genomic fragment contained the first exon common to the two cDNAs (exon 3), starting from the sequence immediately downstream to the myristylation consensus, covering the 14 codons specifying uncharged amino acids of the membrane anchor, and extending into the cytoplasmic domain of the reductase (until position 186 of L-cDNA). The sequence of this exon corresponds to the one identified as the second exon by Zenno et al. (1990), and is not presented in the figure.

To investigate whether the regions of the gene upstream of our cDNA clones were promoters, we mapped transcriptional initiation sites by S_1 nuclease digestion experiments, after hybridization of single-stranded genomic probes with liver or reticulocyte polyA⁺ RNA (Fig. 3, D and E). For exon 1 (Fig. 3 D), we found five major protected fragments, corresponding to transcriptional initiation sites in the region between 2- and 25-bp upstream of the start of our L-cDNA clone (shown in bold-faced letters marked with dots in the sequence of Fig. 3 B). Multiple transcriptional initiation sites are typical of TATA-less promoters (Dyana, 1986). For exon 2 (Fig. 3 E), two specifically protected fragments were observed, (corresponding to the positions shown in bold face and marked with dots in Fig. 3 C), 1- and 3-bp upstream of the start of the R-cDNA clone. It is probable that the smaller protected fragment was due to a "nibbling" artefact, and that the true transcriptional initiation site is at position -3 relative to the start of the cDNA clone, 28-nts downstream from the TATA box.

Tissue Distribution of R- and L-Reductase Transcripts

The similarity of the putative promoter region upstream of the R-specific exon (exon 2) to promoters of erythroid-specific genes (Evans et al., 1990) prompted us to study the expression of R- and L-mRNA in different tissues (Fig. 4). To detect even low levels of each transcript, we used the sensitive PCR technique, under conditions which have been reported to be quantitative (Chelly et al., 1990). 1st strand reductase cDNA, synthesized from heart, brain, skeletal muscle, kidney, liver, or reticulocyte RNA, was amplified with a 3' primer common to the two transcripts, and a 5'

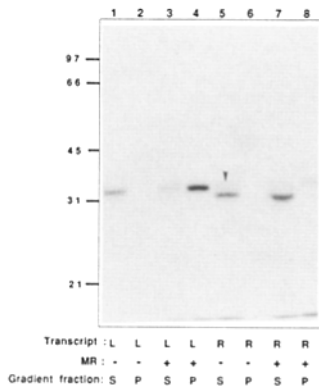


Figure 5. R reductase mRNA generates 2 products in vitro, of which the major one is soluble. L-cDNA (pGbk3, Fig. 1) or R-cDNA (pGbk4, Fig. 1) were transcribed, capped, and translated in vitro in the presence of [³⁵S]methionine. After translation was terminated, the samples were incubated for 30 min with or without rat liver microsomes. The membrane-associated reductase was separated by sedimentation through an alkaline

sucrose cushion (see Materials and Methods for details). The figure shows the result of the analysis of the pellets (P) and supernatants (S) of these gradients by SDS-PAGE (10%) and fluorography. Lanes 1-4, L-cDNA; lanes 5-8, R-cDNA; lanes 1, 2, 5, and 6, posttranslational incubation with no added microsomes; lanes 3, 4, 7 and 8: posttranslational incubation with added microsomes; lanes 1, 3, 5, and 7: gradient supernatants; lanes 2, 4, 6, and 8: gradient pellets. Numbers on the left indicate relative molecular masses and positions of BioRad low molecular weight standards. Downward pointing arrowhead in lane 5 indicates the minor "large" product of R-cDNA, which binds to microsomal membranes (lane 8).

primer specific either for exon 1 or for exon 2 (Fig. 4 A). The amplified reductase cDNAs were detected by Southern blotting. In this experiment, no internal standardization was included, because we were interested in the ratio of L- to R-cDNAs within the same cDNA samples, and not in comparing absolute levels of reductase expression between different tissues. In heart, brain, skeletal muscle, and kidney, we could detect only the L form of reductase cDNA (Fig. 4 B, lanes 1-8). Even with much longer exposures than the one shown in Fig. 4 B, the lanes containing material amplified with the exon 2 specific primer remained negative. In liver, a small amount of R-cDNA was detected (Fig. 4 B, lane 10), but the predominant form was of the L type (lane 9). Only in reticulocytes was the R-form expressed predominantly over the L-form. Thus, the R-mRNA has a pattern of expression consistent with that expected for an erythroid-specific gene, while the L-form has a distribution expected for a housekeeping gene. The small amount of R-cDNA detected in liver may be explained by the large amount of blood in liver homogenates and/or by some hepatic hemopoiesis occurring in the young rats (<1-mo old), which were used in this experiment.

Reticulocyte mRNA Generates the Soluble Form of Reductase from an Internal Initiation Codon

To investigate whether R-mRNA codes for soluble reductase, we analyzed the in vitro translation products of synthetic L- and R-mRNAs. Plasmids suitable for in vitro transcription were constructed (see Materials and Methods), and the synthetic, capped transcripts were translated in a reticulocyte lysate system (Fig. 5). Translation of L-mRNA (derived from pGbk3, Fig. 1) generated a single polypeptide with the known migration of the membrane form of the reductase on SDS-polyacrylamide gels (M_r app \approx 33,000; Fig. 5, lane 1). This polypeptide could be immunoprecipitated by antireductase antibodies, and contained covalently

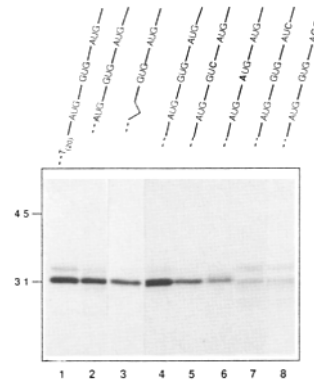


Figure 6. The two in vitro translation products of R reductase mRNA are generated from alternative initiation codons. Capped, synthetic transcripts of wild-type R-cDNA, or mutants thereof were translated in a nuclease-treated reticulocyte lysate in the presence of [³⁵S]methionine. The products were analyzed on a 10% PA gel in SDS. The constructs (see Fig. 1 for details) are schematized at the top of the lanes: the dashed line indicates sequence contributed by the plasmid (between the Sp6 promoter and the cloning site of the reductase cDNA); the full line represents reductase sequence. Lane 1, wild-type R-cDNA, containing the T₍₂₀₎ tail introduced for cloning with anchored PCR (pGbk4); lane 2, T₍₂₀₎ tail and 5' non-coding sequence deleted (pGbk5); lane 3, same as construct of lane 2, but first AUG (boxed in Fig. 2 B) also deleted (pGbk6); lane 4, wild-type R-cDNA without T₍₂₀₎ tail (pGbk9); lane 5, same as construct of lane 4, but with first GUG (underlined in Fig. 2 B) changed to GUC (pGbk11); lane 6, same as construct of lane 4, but with first GUG changed to AUG (pGbk13); lane 7, same as construct of lane 4, but with second AUG (boxed in Fig. 2 B) changed to AUC (pGbk10); lane 8, same as construct of lane 4, but with second AUG changed to AGC (pGbk14). Numbers on the left indicate relative molecular masses and positions of Bio-Rad low molecular weight standards.

linked myristic acid (results not shown). After posttranslational incubation with microsomes most of this form of the reductase was found associated with the membranes in an alkali-resistant manner (Fig. 5, lane 4). Translation of R-mRNA (transcribed from pGbk4, Fig. 1) yielded two polypeptide products (Fig. 5, lane 5): one minor product, with an electrophoretic migration slightly slower than that of the product of L-mRNA (Fig. 5, lane 5, arrowhead); and one major, more rapidly migrating product (apparent molecular weight 31,000), with a position on the gel expected for the soluble form of the reductase (Yubisui et al., 1984). Both these polypeptides were immunoprecipitated with antireductase antibodies (not shown). After posttranslational incubation with microsomes, much of the minor product was found associated with the membranes in an alkali-resistant manner (Fig. 5, lane 8), while all of the major product remained soluble (Fig. 5, lane 7). The interaction of the minor product with membranes was not surprising, since the predicted NH₂-terminal amino acid sequence is quite hydrophobic (see Fig. 2).

These results demonstrated that, at least in vitro, the soluble form of the reductase was generated from R-mRNA, however, the mechanism of its biosynthesis was not clear. It was possible that the soluble reductase was the product of proteolytic processing of the minor 33-kD form. However, since the first AUG of R-mRNA is not in a favorable context for initiation (Pu at -3, G at +4; Kozak, 1989) (see Fig. 2), it seemed likely that R-mRNA is bifunctional, with initiation occurring inefficiently at the first AUG and at an additional downstream start codon (Kozak, 1986). To test this idea, we constructed mutant forms of R-cDNA (see Materials and Methods).

As shown in Fig. 6, when the entire 5' non-coding region

linked myristic acid (results not shown). After posttranslational incubation with microsomes most of this form of the reductase was found associated with the membranes in an alkali-resistant manner (Fig. 5, lane 4). Translation of R-mRNA (transcribed from pGbk4, Fig. 1) yielded two polypeptide products (Fig. 5, lane 5): one minor product, with an electrophoretic migration slightly slower than that of the product of L-mRNA (Fig. 5, lane 5, arrowhead); and one major, more rapidly migrating product (apparent molecular weight 31,000), with a position on the gel expected for the soluble form of the reductase (Yubisui et al., 1984). Both these polypeptides were immunoprecipitated with antireductase antibodies (not shown). After posttranslational incubation with microsomes, much of the minor product was found associated with the membranes in an alkali-resistant manner (Fig. 5, lane 8), while all of the major product remained soluble (Fig. 5, lane 7). The interaction of the minor product with membranes was not surprising, since the predicted NH₂-terminal amino acid sequence is quite hydrophobic (see Fig. 2).

As shown in Fig. 6, when the entire 5' non-coding region

of R-cDNA was deleted, but the first AUG was retained, both forms of reductase were still synthesized (compare lanes 1 and 2). However, when the first AUG was deleted, only the low molecular weight, soluble, reductase was made (lane 3). This result demonstrates that the soluble reductase is not a product of proteolytic processing of the 33-kD form, and that the latter is generated from the first, out-of-context, AUG.

Our next goal was to identify the downstream initiation codon used for the generation of soluble reductase. There are no further AUGs in the R-specific sequence. The first downstream AUG is in the common region, and specifies the last amino acid of the stretch of 14-uncharged residues of the membrane form of the reductase (boxed ATG at position 88 in Fig. 2 B). Thus, initiation from this codon would leave out the entire membrane anchor from the translation product. We also considered the possibility that a GUG codon, within the common region, encoding a Val at the beginning of the hydrophobic stretch, could serve as initiation codon (position 49 of R-cDNA; underlined in Fig. 2 B). This is because it is known that GUG codons can function as initiators, at least in vitro (Peabody, 1989), and because in the human reductase this GUG is substituted with an AUG (Tomatsu et al., 1989). It will be noticed that neither the GUG nor the downstream AUG codon are in a favorable sequence context for initiation, although the sequence surrounding the GUG is slightly more favorable (G at position +4) than the one surrounding the AUG.

When the GUG was mutated to GUC, there was no change in the translation products (compare lanes 4 and 5 of Fig. 6). On the other hand, when this GUG was mutated to AUG, a third product appeared, with slightly slower mobility than that of the soluble reductase (Fig. 6, lane 6). Therefore, the soluble reductase is not generated from the GUG at position 49. In contrast, when the downstream AUG was changed to AUC, there was a dramatic decrease in the amount of soluble reductase produced (Fig. 6, lane 7), indicating that this downstream AUG is the initiator codon for the soluble reductase. A small amount of soluble reductase was still produced even after mutation of the AUG to AGC (Fig. 6, lane 8). This might be explained by the use of the two immediately adjacent downstream codons, AAG and CUG, both with an A at position -3, and both of which have been shown to function in vitro (Peabody, 1989).

It is known that initiation codons used in cell-free translation systems often do not reflect the in vivo situation (Kozak, 1989). To rule out the possibility of artifacts due to the reticulocyte cell-free system, we investigated the in vivo translation of reductase transcripts, by microinjecting *Xenopus* oocytes with the same synthetic capped RNAs used for the in vitro experiments. After pulse labeling the injected oocytes, reductase was immunoprecipitated from the cytoplasmic and membrane fractions (Fig. 7). The L-mRNA generated a reductase product with the same gel migration as the in vitro synthesized form (compare lanes 4 and 5 of Fig. 7). Nearly all of this reductase associated with membranes (compare lanes 3 and 4 of Fig. 7). It was more difficult to detect the translation product of microinjected R-mRNA. However, after a long exposure of the gel, it was possible to see a polypeptide, comigrating with the in vitro synthesized soluble reductase, specifically immunoprecipitated from the soluble fraction of the oocyte (lane 8). This band was not present in the membrane fraction (lane 9), and thus corre-

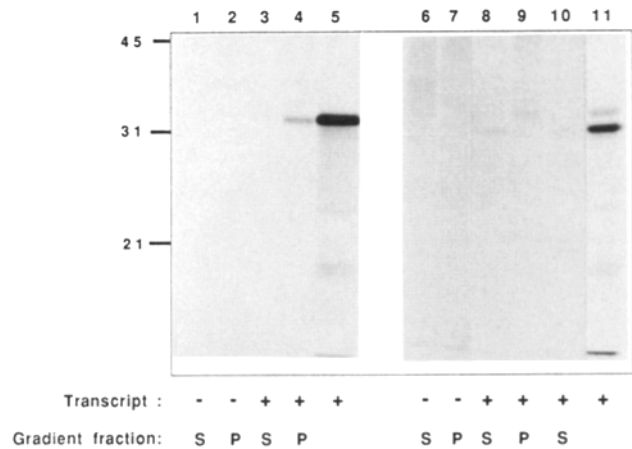


Figure 7. Translation of reductase mRNAs in *Xenopus* oocytes. Capped transcripts generated from pGbk3, pGbk4, or pGbk6 (Fig. 1) were injected into *X. laevis* oocytes. After incubation for 2 h with [³⁵S]methionine, the oocytes were homogenized and separated into membrane (P), and soluble (S) fractions. Fractions were solubilized and immunoprecipitated with antireductase antibodies (see Materials and Methods for details). The figure shows the result of SDS-PAGE (11%)-fluorography analysis of the immunoprecipitates. Lanes 1, 2, 6, and 7, control, mock-injected oocytes; lanes 3 and 4, oocytes injected with L-mRNA (pGbk3); lanes 8 and 9, oocytes injected with R-mRNA (pGbk4); lane 10, oocytes injected with mutant R-mRNA, lacking the first in frame AUG codon (pGbk6). Lanes 1, 3, 6, 8 and 10, soluble fraction of oocyte; lanes 2, 4, 7, and 9, membrane fraction of oocyte. Lanes 5 and 11 show the in vitro translated products (not immunoprecipitated) from L-mRNA (pGbk3) and R-mRNA (pGbk4), respectively. Immunoprecipitates from oocyte fractions were derived from 4.3 oocytes, except for samples of lanes 3 and 4, which were derived from half that amount. For the in vitro samples, 1 μ l of translation, containing transcript deriving from \approx 1 ng of plasmid, was loaded. Lanes 1-5 and 11 were exposed overnight; lanes 6-10 were exposed for 12 d. Numbers on the left indicate relative molecular masses and positions of Bio-Rad low molecular weight standards.

sponds to soluble reductase. The soluble reductase was also generated from the mutant R-mRNA with deletion of the first AUG (lane 10). These results confirm those obtained with in vitro translation. We were also interested in knowing whether the minor NH₂ terminally extended product is translated from mRNA in vivo. However, after long periods of exposure, products immunoprecipitated from the membrane fraction of noninjected oocytes became visible on the fluorogram in the 30-kD region (lane 7), so that it was not possible to evaluate the significance of the 33-kD polypeptide in the immunoprecipitate obtained from the membrane fraction of oocytes injected with R-mRNA (lane 9).

Discussion

Fig. 8 illustrates how a combination of mechanisms operating at both the transcriptional and translational levels generates different isoforms of the reductase in a tissue-specific fashion, as indicated by the results of this paper. In brief, the first exon of the reductase gene contains a 5'-untranslated sequence and an ATG in a favorable context for translational initiation, followed by six codons which specify the myristylation consensus. Exon 1 is preceded by a region with fea-

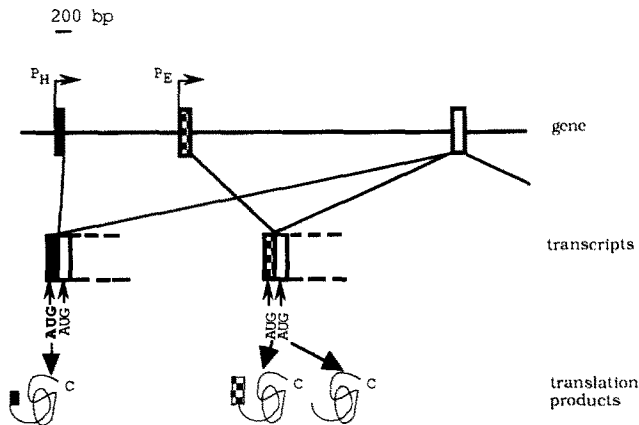


Figure 8. Organization of 5' portion of reductase gene: generation of two transcripts and three translation products. In the top line, which represents the gene, the symbols indicate: P_H, housekeeping promoter; P_E, erythroid promoter; ■, exon encoding myristylation consensus; ▨, exon encoding reticulocyte-specific hydrophobic sequence; □, first common exon. In the middle line, representing the transcripts, the AUG in bold-faced letters indicates a strong initiation codon, while the two AUGs in normal lettering indicate weak initiation codons. The third line depicts the protein products differing at the NH₂-termini: protein with NH₂-terminal filled rectangle: myristylated version of the reductase; protein with NH₂-terminal checkered rectangle: reticulocyte product with hydrophobic, non-myristylated NH₂-terminus; product without NH₂-terminal rectangle: soluble reductase. See text for further explanation.

tures common to promoters of house-keeping genes, consistent with the expression of the myristylated membrane form of reductase in a variety of tissues. Exon 2 contains a 5'-untranslated region and an AUG in an unfavorable context for translational initiation, followed by 12 codons specifying uncharged amino acids. It is flanked by a region with features common to erythroid promoters, consistent with the tissue-specificity reported here for the expression of R-mRNA, and with the known erythroid localization of the soluble reductase. Transcription from this promoter excludes exon 1 from R-mRNA, which instead codes for 2-nonmyristylated forms of the reductase; one, starting from the first AUG, with a hydrophobic NH₂ terminus; the second one starting from a downstream AUG, which lacks the entire membrane anchor and which corresponds to soluble reductase. Both these forms are produced in the reticulocyte lysate, while up till now we have been able to demonstrate only the production of the soluble form in vivo. The downstream AUG codon, used to generate soluble reductase, is present also in the exon 1-containing transcript, where it is not used. Several features of the model of Fig. 8 deserve discussion.

Organization of the Reductase Gene

The cotranslational addition of myristic acid to the NH₂-terminal glycine of proteins via an amide bond has, in addition to the absolute requirement for gly at position 2 of the primary translation product, other requirements for the residues immediately downstream to this position (Gordon et al., 1991). Positions 3 and 6 are of particular importance, with only small, uncharged residues allowed in position 3, and Ser in position 6 favoring a low *K_m* for the N-myristoyl-transferase. Based on experiments with synthetic peptides, a core hexapeptide signal sequence for myristylation has been

defined (Towler et al., 1988). In agreement with these studies, expression of clones coding for mutant forms of p60^{v-src} (Kaplan et al., 1988) has implicated the first seven residues of the primary translation product as constituting the myristylation signal, while studies with chimaeras have shown that the first six codons of p60^{v-src} are sufficient to obtain myristylation of a passenger protein (Buss et al., 1988). The first seven codons of the L-transcript for the reductase conform perfectly to the consensus sequence for myristylation. Thus, it seems reasonable to conclude that all the information necessary for myristylation is contained in a separate exon (exon 1) of the reductase gene, and that this exon contains coding information only for the myristylation signal sequence. This segregation of the information for myristylation in a separate exon has not been found in the genes for other cellular myristylated proteins, i.e., in the chicken *c-src* gene (Takeya and Hanafusa, 1983), in the human *lck* gene (Garvin et al., 1988), or in the gene for human MARCKS (Harlan et al., 1991). However, the segregation of signal sequences in separate exons is found for other NH₂-terminal signals, such as the one for translocation across the ER. Thus, it is possible that the myristylation signal will turn out to be separated from the downstream coding sequences also in other genes, with obvious evolutionary and regulatory implications.

Both the human (Tomatsu et al., 1989) and the rat (Zenno et al., 1990) reductase gene had been previously cloned and reported to consist of nine exons. Here, we demonstrate the presence of a 10th exon in the rat gene, in between the first two exons found by those authors, which codes for the sequence unique to the R transcript. Both exon 1 and exon 2 were preceded by promoter-like regions. That these regions both function as promoters is suggested by the results of the S1 nuclease protection experiments, since the 5' extremities of the protected fragments mapped to positions within the gene not corresponding to 3' splice sites, and therefore presumably corresponding to transcriptional initiation sites. The two putative promoters have different characteristics: the one preceding exon 1 has features common to promoters of housekeeping genes (Dynam, 1986), while the regions flanking exon 2 have several possible *cis*-regulatory elements found in erythroid promoters, i.e., a TATA box at the expected distance from the cap site, CAAT-like sequences, and, notably, two binding sites for the erythroid-specific transcription factor GATA-1, one of them close to a "GT" box. Such *cis*-acting regulatory elements, are found in globin promoters (Evans et al., 1990), as well as in those of non-globin erythroid genes, such as the glycophorin C (Van Kim et al., 1989) the erythroid porphobilinogen deaminase (Chretien et al., 1988), and the erythroid 5-aminolevulinatase synthase (Cox et al., 1991) genes. The pattern of expression of the R-type reductase mRNA was also consistent with the erythroid tissue specificity of the promoter. Thus, we conclude that the two transcripts, the ubiquitous L-mRNA, and the tissue-specific R-mRNA, are generated by an alternative promoter mechanism, involving an upstream housekeeping promoter, and a downstream erythroid promoter. A similar situation has been reported for the human porphobilinogen deaminase gene (Chretien et al., 1988), while the erythroid-specific isoenzyme of rat pyruvate kinase is transcribed from an alternative upstream promoter (Noguchi et al., 1987). In these examples, however, the erythroid-specific protein has the same cytoplasmic localization and the same function as the protein expressed elsewhere. In the case of the reductase,

the use of an alternative promoter generates an mRNA whose major product is different from that produced elsewhere with respect to modification of the primary structure (no myristylation), localization, and function.

Translation of R-type Reductase mRNA

The two *in vitro* translation products of R-mRNA were shown to originate from two AUG codons, both in an unfavorable context for initiation (i.e., a pyrimidine in position -3, and a non G at position +4, relative to the A of the initiation codon; Kozak, 1989). The use of a downstream AUG as initiation codon, when the first AUG is weak (leaky scanning), is predicted by the scanning model (Kozak, 1986). We conclude that the major translation product, generated from the downstream AUG, corresponds to the well-known soluble form of the reductase, because: (a) it was produced both *in vivo* and *in vitro* and remained soluble in both cases; (b) it migrated on SDS-PA gels with the expected mobility, and the downstream initiator AUG codon is close (three codons upstream) to the UUU codon, which specifies the Phe reported to be the NH₂-terminal residue of the purified human soluble reductase (Yubisui et al., 1986).

Although we could not detect the minor NH₂-terminally extended form of the reductase in microinjected oocytes, we believe that it is produced *in vivo*, because it starts from the first AUG, and in all known cases the first AUG is used for initiation of translation; a suboptimal sequence context will drastically lower recognition by the small ribosomal subunit, but is not expected to completely abolish initiation (Kozak, 1989). At present we don't know anything about the *in vivo* localization and function of this minor, higher molecular weight previously undescribed form of the reductase. Mammalian erythrocytes do have plasma membrane-bound reductase (Borgese et al., 1982; Choury et al., 1981), which was always assumed to be identical to the myristylated reductase expressed elsewhere and posttranslationally delivered to ER and outer mitochondrial membranes (Borgese and Gaetani, 1980, 1983; Borgese and Pietrini, 1986). It is possible that the plasma membrane reductase of red cells corresponds to the nonmyristylated form described here, and that the nonmyristylated hydrophobic NH₂-terminal sequence is related to its different localization in red cells. A receptor for the myristoyl-NH₂-terminal peptide of p60^{src} appears to be involved in the plasma membrane localization of that protein (Resh and Ling, 1990). It could be that a similar receptor, specific for the myristoyl-NH₂-terminal peptide of the reductase, is involved in its endoplasmic reticulum and outer mitochondrial membrane localization. It should also be mentioned that analysis of the NH₂-terminal sequence of NH₂-terminally extended, non-myristylated, isoform of the reductase with the von Heijne algorithm (von Heijne, 1986) gave a score of +4, 26 for cleavage between ser-15 and arg-16. This value is well within the range of scores found for translocated proteins, and raises the interesting possibility that the use of the erythroid alternative promoter results in the swapping of a myristylation signal sequence for an ER translocation sequence. We are now attempting to characterize the biosynthesis and interaction with membranes of this interesting product both *in vivo* and *in vitro*.

As already mentioned, the production of two polypeptide products from the R transcript is consistent with the scanning model, which predicts that an mRNA will be bifunc-

tional if the first initiation codon is in an unfavorable context. Only some of the small ribosomal subunits will recognize it, while others will scan further and initiate at a downstream codon (Kozak, 1986). Also consistent with this interpretation of our data is the fact that the downstream initiation codon used for the synthesis of soluble reductase is present in the ubiquitously expressed transcript, but is not used, a phenomenon which can be explained by the presence of a strong first AUG, which prevents any further scanning of the small ribosomal subunit. Indeed, we found that if the context of the first AUG of L-mRNA was made suboptimal, the downstream AUG was used (results not shown). Unmasking of a downstream AUG by an alternative promoter mechanism is a common ploy for the production of a short and long version of the same protein (examples listed in Kozak, 1991). In the case of the reductase, however, use of the alternative promoter results in swapping of a strong AUG for a weak one, with two effects: the production of a new, NH₂-terminally extended form of reductase, and the unmasking of the downstream AUG codon.

A number of viral and cellular bifunctional messengers have been recognized (listed in Kozak, 1991), some of which generate products with different subcellular localization (Bugler et al., 1991; Lock et al., 1991) or with different modification of their primary structure (Kaminchik et al., 1991). In most bifunctional mRNAs, however, the downstream initiation context is in a favorable context, or, at least, has a purine in position -3. In the case of the reductase, also the downstream AUG is in an unfavorable context, and its relatively high efficiency of utilization relative to the upstream AUG may be due to secondary structural features in its surroundings, or to its greater distance from the cap site. Nonetheless, we expect that both these AUGs are used inefficiently *in vivo*, as suggested also by our results with microinjected *Xenopus* oocytes. R-mRNA may belong to a class of transcripts which seem designed for poor translation (Kozak, 1991), and which often code for regulatory proteins, such as transcription factors and protein kinases. The concentration of soluble reductase in the erythrocyte is low (Borgese et al., 1982), although the level of mRNA seems close to that found in liver (Pietrini et al., 1988 and Fig. 3 of this paper), an organ which has a considerably higher concentration of the protein. This discrepancy between mRNA and protein concentration might be explained by inefficient reductase synthesis ensured by a poorly translatable messenger.

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