

Tissue-specific translational regulation of alternative rabbit 15-lipoxygenase mRNAs differing in their 3'-untranslated regions

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Received January 18, 1999; Revised and Accepted February 19, 1999

DDBJ/EMBL/GenBank accession nos*

ABSTRACT

By screening a rabbit reticulocyte library, an alternative 15-LOX transcript of 3.6 kb (15-LOX mRNA2) was detected containing a 1019 nt longer 3'-untranslated region (UTR2) than the main 2.6 kb mRNA (15-LOX mRNA1). In anaemic animals, northern blotting showed that 15-LOX mRNA2 was predominantly expressed in non-erythroid tissues, whereas 15-LOX mRNA1 was exclusively expressed in red blood cells and bone marrow. The 15-LOX 3'-UTR2 mRNA2 contained a novel 8-fold repetitive CU-rich motif, 23 nt in length (DICE2). This motif is related but not identical to the 10-fold repetitive differentiation control element (DICE1) of 19 nt residing in the 15-LOX UTR1 mRNA1. DICE1 was shown to interact with human hnRNP proteins E1 and K, thereby inhibiting translation. From tissues expressing the long 15-LOX mRNA2, two to three unidentified polypeptides with molecular weights of 53–55 and 90–93 kDa which bound to DICE2 were isolated by RNA affinity chromatography. A 93 kDa protein from lung cytosol, which was selected by DICE2 binding, was able to suppress translational inhibition of 15-LOX mRNA2, but not of 15-LOX mRNA1, by hnRNP E1. A possible interaction between DICE1/DICE2 *cis/trans* factors in translational control of 15-LOX synthesis is discussed. Furthermore, the 3'-terminal part of the highly related rabbit leukocyte-type 12-LOX gene was analysed. Very similar repetitive CU-rich elements of the type DICE1 (20 repeats) and DICE2 (nine repeats) were found in the part corresponding to the 3'-UTR of the mRNA.

INTRODUCTION

Mammalian lipoxygenases (LOX) are classified into 5-, 8-, 12- and 15-LOXs, according to the positional specificity by which they catalyse the oxygenation of the substrate, arachidonic acid. 5-LOX is involved in the synthesis of leukotrienes acting as

mediators of inflammatory and anaphylactic reactions (1). 15-LOX has been implicated in red blood cell maturation (2) and in the process of atherogenesis (3,4). For 12-LOXs, three different independent isoforms have been described, which are coded by different genes. The platelet-type 12-LOX (5) is involved in platelet aggregation (6), whereas functions for the leukocyte-type (7), the epidermal-type 12-LOX (8) and the phorbol ester-inducible 8-LOX (9) are not well defined. Enzymology and molecular biology of mammalian LOXs has been reviewed (10,11).

15-LOX is expressed at a high level in reticulocytes (12) and airway epithelial cells (13), but can also be detected in a variety of other tissues (14,15). 15-LOX mRNAs have to date only been cloned from rabbit (16) and man (17). In contrast, other species including mouse, pig and cow seem to express predominantly a leukocyte-type 12-LOX. This finding suggested a functional equivalence of reticulocyte 15-LOX and leukocyte-type 12-LOX in different species (18). However, we recently showed the simultaneous expression of 15-LOX and leukocyte-type 12-LOX in rabbits, which supports the idea of an independent physiological function for these two isoenzymes (19).

The tissue-specific expression of 15-LOX is highly regulated at the transcriptional and post-transcriptional levels (20). Bleeding, phenylhydrazine administration or cholesterol feeding results in 15-LOX synthesis induction (21). In monocytes, 15-LOX synthesis is controlled transcriptionally by IL-4 and γ -interferon (22). During erythroid differentiation, 15-LOX synthesis is regulated translationally by interaction of specific repressor proteins (LOX-BP) with a repetitive CU-rich motif, called DICE (differentiation control element). DICE reside in the 3'-untranslated region (3'-UTR) of the mRNA (23). Two lipoxygenase mRNA binding proteins (LOX-BP) involved in cytoplasmic mRNA translation have been identified as the nuclear hnRNP proteins E1 and K (24). Although the phenomenon of 15-LOX induction is well known (2,21) molecular details have hardly been investigated. In this paper, we show that anaemia induction results in the formation of two different 15-LOX transcripts differing in their 3'-UTRs. They have different tissue specificities and are formed by alternative polyadenylation. The longer 3'-UTR of the

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+M27214, AJ003023, AJ003024

non-erythroid 15-LOX mRNA harbours a novel CU-rich repetitive motif related to DICE. Its protein-binding properties and involvement in translational regulation are investigated.

The genomic organisation of mammalian arachidonate lipoxygenase genes (*Alox*) is highly conserved. All genes analysed so far constitute 14 exons interrupted by 13 introns (11). The genes span between 7.3 kb in the case of the mouse *Alox1* gene (25) and >82 kb for the human *Alox5* gene (26). We have published the complete sequence of the rabbit 8.0 kb long *Alox15* gene (27). Because alternative transcripts have been described, we now present a revised model of this gene. Furthermore, we show data on the structure of the hitherto unknown rabbit leukocyte-type *Alox12* 3'-terminal gene region.

MATERIALS AND METHODS

Rabbit reticulocyte cDNA and genomic DNA library screening for 15-LOX cDNAs and the *Alox15* gene

Lipoxygenase cDNA clones were isolated by screening a rabbit reticulocyte custom library (Stratagene) obtained by cloning poly(A)⁺ RNA from rabbits (adult Chinchilla crossbreeds) made anaemic by bleeding (28) into the *EcoRI/XhoI* arms of the λ ZAP Express vector. The library contained 2.4×10^6 p.f.u. primary plaques. As a hybridisation probe a ³²P-labelled rabbit 15-LOX cDNA was used (16). LOX-positive primary plaques were purified by two to three rounds of screening. For further manipulation and sequencing, the recombinants were excised *in vivo* as pBK-CMV plasmids as described in the Stratagene protocol. LOX plasmids were subjected to conventional restriction enzyme and PCR mapping techniques to detect length differences to the main 2.6 kb 15-LOX mRNA (16).

Genomic recombinants of the rabbit 15-LOX gene were obtained by screening a commercially available rabbit genomic phage library in the vector λ DASH II (Stratagene 945950) as described above. The DNA used for cloning was a *Sau3AI* partial digest from heart of 2–14-day-old New Zealand Whites and cloned into the *BamHI* site of the vector. For further analysis, *EcoRI-XhoI* fragments of the *Alox15* gene inserts were subcloned into the Bluescript SK plasmid vector (Stratagene). Double-stranded sequencing was performed by the dideoxy chain termination method with Sequenase v.2.0 (US Biochemical Corp.) or by cycle sequencing with fluorescence labelled nucleotides using the ABI sequencer, model 373.

Genomic PCR cloning of 12-LOX genomic sequences

For the amplification of rabbit *Alox12l* gene fragments, two 12-LOX-specific primers were designed. They correspond to nucleotides 2438–2458 of the 1.12-LOX cDNA (19). The upstream primer (LX10H) has the sequence 5'-CGCCGAC-CTCCTAAGGCGTCA-3' and the downstream primer (LX10R) 5'-TGACGCCTTAGGAGGTCGGCG-3'. These primers were combined with primers common to 12-LOX and 15-LOX exons 10 (LX1327H, 5'-GGAGCCTTCTTAACCTATCG-3') or 14b (LX-1.5X-R7, 5'-CTCTGCAAGCCTGTTTGTGA-3').

For genomic PCR, 500 ng of rabbit liver DNA isolated with a Nucleon DNA extraction kit from Scotlab Biosciences (Wiesloch, Germany) were used in a 25 μ l assay (PCR kit; InViTek, Berlin, Germany). Amplification was performed with a Trioblock from Biometra (Göttingen, Germany). PCR products were subcloned

into the plasmid vector pCR II (Invitrogen, ITC Biotechnology GmbH, Heidelberg, Germany) and sequenced as described above.

Cloning and expression of rabbit hnRNP proteins E1 and K

A rabbit reticulocyte cDNA library (see above) was screened with rabbit-specific hnRNP E1 and hnRNP K cDNA PCR fragments. These were generated by amplification of rabbit-specific sequences from the library by use of human-specific primers selected from the published sequences for hnRNP E1 (29) and hnRNP K (30). The following primer pairs were used: 5'-ATCATTGGGAAG-AAAGGGGAGTCG-3' and 5'-ATGGGCTGGTACGGAATG-GTCA-3' for hnRNP E1; 5'-CTCCCGCTCGAATCTGATGCT-GTGG-3' and 5'-GGTGGTGGTGGAGGAAGAGGAAGAT-3' for hnRNP K. Positive phage clones were excised as pBK-CMV plasmids and sequenced as described above.

For bacterial expression the coding sequences were cloned in-frame into the expression vector pET-22b (Novagen) for transformation of *Escherichia coli* BL21 (DE3). A hnRNP E1/rab *NcoI-HindIII* fragment was ligated into *NcoI/HindIII*-digested pET-22b. To create a novel 5' *NcoI* site in the hnRNP K/rab cDNA sequence the following PCR primers were used: 5'-ACAAGAC-CATGGAACTGAACAGCCGGAGG-3' (forward) and 5'-GGT-GGTGGTGGAGGAAGAGGAAGAT-3' (reverse). The resulting PCR fragment was restricted by *NcoI* and *HindIII* (534 bp) and ligated into *NcoI/HindIII*-cut pET-22b. This partial cDNA clone was completed by addition of the *HindIII-XhoI* fragment of pBK-CMV-K/rab (1319 bp).

Transformed bacteria were grown in LB medium at 37°C and after 3 h expression of recombinant proteins was induced by addition of IPTG (0.4 mM final concentration) for another 3 h. The cells were pelleted and lysed in 50 mM Tris pH 8.0, 2 mM EDTA, 0.1 mg/ml lysozyme, 0.1% Triton X-100 for 15 min at 30°C and sonicated for 2 min with a Branson sonifier in the presence of 0.1% PMSF and 10 mg/ml leupeptin. After centrifugation at 12 000 g for 15 min at 4°C, hnRNP proteins E1 and K were isolated by RNA affinity purification on biotinylated LOX 3'-UTR transcripts (10 repeats) and streptavidin-agarose beads as described (22).

RNA blot analysis

Total RNA of various rabbit tissues, frozen in liquid nitrogen, was isolated by the guanidine thiocyanate method after the manufacturer's protocol (RNA-Clean; AGS, Heidelberg, Germany). Poly(A)⁺ RNA was isolated by batch-wise binding to poly(U)-Sephrose (Pharmacia, Uppsala, Sweden) as described (12). Samples of 1.0 μ g poly(A)⁺ RNA were electrophoresed in 2.2 M formaldehyde–1.2% agarose gels after denaturation in DMSO/formamide and transferred to nylon membranes (Hybond N; Amersham Buchler, Braunschweig, Germany) according to the protocol of the manufacturer. Filters were hybridized with a ³²P-labelled 15-LOX cDNA probe (16).

Distribution of 15-LOX in rabbit tissues by immunoblotting

Tissues of normal animals or animals made anaemic by bleeding (28) were homogenised with a knife homogeniser (Ultraturrax) in a buffer containing 10 mM HEPES, pH 7.2, 1.5 mM MgCl₂, 10 mM LiCl, 0.5 mM DTT, 0.5 mM PMSF, 1 mM pepstatin A and 1 mM leupeptin (1 vol tissue/1.5 vol buffer). The homogenate was centrifuged twice at 10 000 g for 10 min. The supernatant

containing 10–40 mg protein/ml was directly used for immunoblotting. An aliquot of 30 µg protein mixture per tissue and slot was separated by 15% SDS-PAGE, electroblotted onto Immobilon P membranes (Millipore) and developed using a guinea pig anti-rabbit reticulocyte 15-LOX polyclonal antiserum as described (31).

HPLC analysis of LOX reaction products

RP-HPLC was carried out on a Nucleosil C-18 column (KS system, 250 × 4 mm, 5 mm particle size; Macherey/Nagel, Düren, Germany). A solvent system of methanol/water/acetic acid (85:15:0.1 v/v/v) and a flow rate of 1 ml/min were used. The absorbance at 235 nm was monitored. For quantification, a calibration curve (five point measurements) for 13-hydroxy-9Z,11E-octadecadienoic acid (conjugated dienes) was established. The fractions containing the oxygenated polyenoic fatty acids were pooled, the solvent was evaporated, the residues were reconstituted in a mixture of *n*-hexane/2-propanol/acetic acid (100:2:0.1 v/v/v) and injected into straight phase and/or chiral phase HPLC columns. Straight phase HPLC (SP-HPLC) of the hydroxy fatty acid isomers was carried out on a Zorbax SIL column (KS system, 250 × 4 mm, 5 mm particle size; Macherey/Nagel, Düren, Germany) with a solvent system consisting of *n*-hexane/2-propanol/acetic acid (100:2:0.1 v/v/v) and a flow rate of 1 ml/min. Chiral phase HPLC was carried out on a Chiralcel OD column (250 × 4.6 mm, 5 mm particle size; Diacel Chemical, distributed by Baker, Groß-Gerau, Germany) with a solvent system of hexane/2-propanol/acetic acid (100:5:0.1 v/v/v) and a flow rate of 1 ml/min. For detection of the hydroxy fatty acids the absorbance at 235 nm was recorded.

UV cross-linking analysis

A 241 nt *BalI*–*HaeII* fragment containing the DICE1 motif of 15-LOX 3'-UTR1 (Fig. 5; DICE1 = 10 repeats) was subcloned into the *SmaI* site of pBluescript and a 328 nt *BglIII*–*AlwNI* fragment containing the DICE2 motif of 15-LOX 3'-UTR2 (Fig. 5; DICE2 = 8 repeats) into the *BamHI* site of the same vector. Plasmids were linearised with *EcoRI* and transcribed with T3 polymerase (DICE1 motif) or with *XbaI* and transcribed with T7 polymerase (DICE2 motif). UV cross-linking analysis using ³²P-labelled transcripts and fractions of RNA-binding proteins was performed as described earlier (23). Briefly, 1–2 ng representing 100 000 c.p.m. [³²P]UTP-labelled transcripts were incubated with 40–120 µg cytosolic protein extract or 50–100 ng recombinant hnRNP proteins E1 or K for 15 min at room temperature in 10 mM HEPES pH 7.2, 3 mM MgCl₂, 5% glycerol and 1 mM DTT, in the presence of 50 µg/ml *E.coli* rRNA in a total volume of 10 µl. Heparin was added to a final concentration of 5 mg/ml. Then the samples were exposed to UV light (255 nm) for 15 min on ice, treated with RNase A (30 µg/ml final concentration) and RNase T1 (750 U/ml final concentration) for 15 min at 37°C and subjected to 15% PAGE and autoradiography.

In vitro transcription/translation

15-LOX cDNAs and 3'-UTR fragments were cloned into the transcription vector pBluescript SK, *in vitro* transcripts were generated using the Stratagene kit and purified by phenol extraction. *In vitro* translation was performed in the rabbit reticulocyte lysate system in the presence of [³⁵S]methionine. All

these techniques have been described in detail earlier (23). In translation inhibition experiments 30–60 ng recombinant proteins E1 and K affinity purified by binding to LOX 3'-UTR1 transcript or rabbit lung protein affinity purified by binding to LOX 3'-UTR2 transcript were added per 12.5 µl assay.

Preparation of polysomes, mRNPs and RNA for RT-PCR

Rabbits were made anaemic by five consecutive daily injections of phenylhydrazine (7 mg/kg) and killed at day 7. Reticulocyte lysate was prepared according to Thiele *et al.* (28). Heart lysate was prepared by homogenising 10 g heart muscle tissue in 30 ml PMS buffer (20 mM HEPES, 250 mM sucrose, 250 mM KCl, 5 mM MgCl₂, 2 mM DTT, 1 mg/ml heparin, pH 7.5) with a knife homogeniser on ice and centrifugation at 4000 g for 10 min. The supernatant was made 1% in Triton X-100 and 1 µl/ml RNasin (Pharmacia) was added. This mixture was centrifuged for 15 min at 10 000 g. Polysomes were pelleted from the supernatant by centrifugation through a 1 M sucrose cushion in PMS buffer for 2 h at 100 000 g. The post-polysomal mRNP fraction consisting of ~3% mRNP particles and 97% ribosomal subunits was sedimented from the 100 000 g supernatant by further centrifugation at 3 000 000 g for 3 h.

Polysomal and mRNP pellets obtained from 5 ml reticulocyte or heart lysate were resuspended in 0.5 ml PUB buffer (0.1 M NaCl, 10 mM Tris, 10 mM EDTA, 1% SDS, pH 7.4) and digested for 20 min at room temperature with 25 ml proteinase K (3 mg/ml). The mixture was extracted once with phenol, phenol/chloroform and chloroform and RNA was ethanol precipitated.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase (RT) reaction. A 14 µl RT reaction contained 1 µg total RNA, 12 pmol each of 18S rRNA and 15-LOX reverse primers (18SR and LX1600R), 4 µl 5× first strand buffer (Gibco BRL), 2 µl 0.1 M DTT, 1 µl 10 mM dNTPs and 1 µl Superscript II revertase (Gibco BRL). Incubation was for 50 min at 42°C and for 15 min at 70°C.

PCR. A 50 µl reaction contained 1 µl RT reaction, 5 µl 10× PCR buffer (InViTek), 3.5 µl MgCl₂ (50 mM), 2 µl of each primer (24 pmol) LX1327H, LX1600R, 18SH and 18SR, 2.5 µl 10 mM dNTPs and 0.5 µl *Taq* polymerase (2.5 U; InViTek). PCR conditions were 1 min at 95°C, 1.5 min at 62°C, 1 min at 72°C for 15–24 cycles (Fig. 7) and 8 min extension at 72°C. Primers: 18SH, 5'-GTC CCC GCC CCT TGC CTC TCG-3'; 18SR, 5'-CCG GTC GGG TCA TGG GAA TAA CG-3'; LX1327H, 5'-GGA GCC TTC TTA ACC TAT CG-3'; LX1600R, 5'-GTG ACA AAG TGG CAA GCC TG-3'.

The plasmid ΔLOX (Fig. 7) was constructed by an in-frame deletion of a 705 bp *PstI* fragment (amino acids 263–497) from a rabbit 15-LOX cDNA plasmid lacking its 3'-UTR as described (23).

DNA sequence data

Novel sequences have been deposited in the EMBL database under the following accession nos: rabbit hnRNP protein E1 cDNA, AJ003023; rabbit hnRNP protein K cDNA, AJ003024. The novel 3'-UTR2 of the rabbit 15-LOX cDNA has been submitted as an update to accession no. M27214.

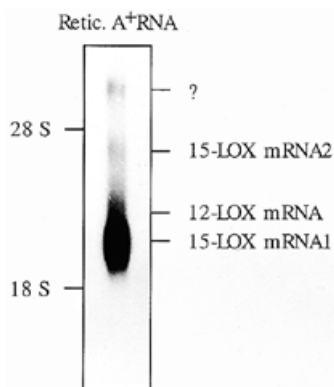


Figure 1. RNA blot analysis of rabbit reticulocyte poly(A⁺) RNA. An aliquot of 1.0 µg rabbit reticulocyte poly(A⁺) RNA was electrophoresed on a formaldehyde-agarose gel, blotted onto Hybond-N and hybridised to a ³²P-labelled rabbit 15-LOX cDNA plasmid probe (16). As a length standard 18S and 28S rRNA were run.

RESULTS

Description of an alternative transcript of the *Alox15* gene

Northern blots of rabbit reticulocyte poly(A)⁺ RNA probed with a segment of the 15-LOX coding sequence show above the main well-characterised 2.6 kb transcript at least three larger minor bands which could represent LOX mRNAs of unknown structure and function or unspliced transcripts (Fig. 1).

To investigate these minor transcripts, a rabbit reticulocyte cDNA phage library was constructed and screened for LOX cDNAs differing from the main 2.6 kb 15-LOX cDNA by plaque hybridisation using a 15-LOX coding probe. Among about 250 LOX-positive clones six clones were found containing inserts >2.6 kb. All were analysed by restriction enzyme mapping and sequencing.

One group of clones represented cDNAs in which the coding region was highly related but not identical to the 15-LOX cDNA. It differed in six amino acids and in the structure of the 3'-UTR. The 2.8 kb cDNA contained a LOX-BP binding motif (differentiation control element, DICE) (23) of 20 repeats instead of 10. Bacterial expression and product analysis identified this protein as a leukocyte-type 12-LOX (19).

A second group of cDNAs represented transcripts of 3.6 kb with a coding and 5'-untranslated region (5'-UTR) identical to the formerly published main 2.6 kb 15-LOX cDNA1 (16) but containing a 1019 nt longer 3'-UTR (3'-UTR2). The sequence of the complete 3'-UTR2 of the 15-LOX cDNA2 has been deposited in the EMBL database as an update to accession no. M27214. The 3'-UTR contains a remarkable motif of eight 23 bp repeats related to the repetitive CU-rich 19 bp DICE motif of 3'-UTR1 which has been shown to mediate translational control in LOX synthesis (23,24). A comparison of the structures of both types of repetitive motifs of 15-LOX mRNAs and those which have been found in the *Alox12* gene (see later) is shown in Figure 3.

In the northern blot (Fig. 1) a third minor signal of ~5 kb is visible. It is unknown if this corresponds to a yet undetected LOX mRNA variant, a partially spliced 12/15-LOX pre-mRNA intermediate or represents a non-specific cross-hybridisation

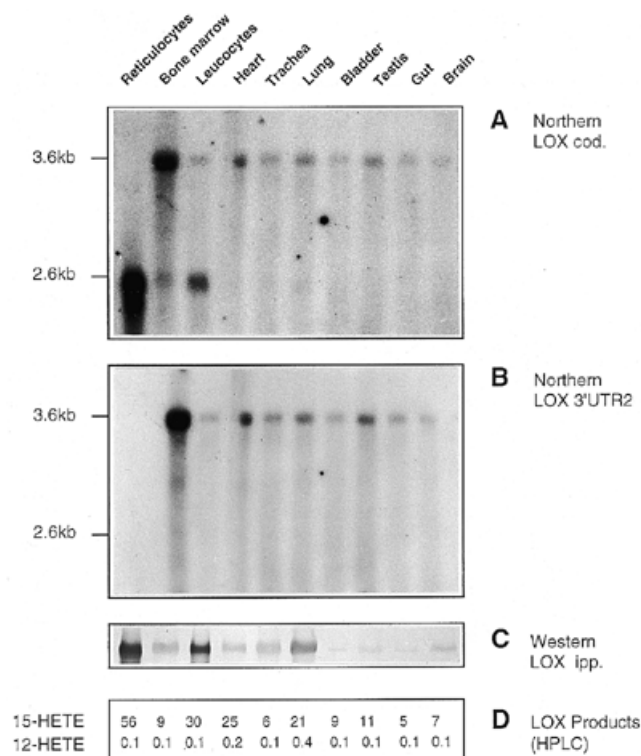


Figure 2. Distribution of 15-LOX mRNAs and protein in rabbit tissues. Poly(A⁺) RNA was isolated from various rabbit tissues of animals made anaemic by bleeding (12), electrophoresed on formaldehyde-agarose gels, blotted onto Hybond-N membranes and hybridised to a ³²P-labelled rabbit 15-LOX coding probe (A) or after stripping to a probe corresponding to the 3'-UTR2 of the rabbit 15-LOX mRNA2 (B). An aliquot of 1 µg of poly(A⁺) RNA was applied per slot. Cytosolic proteins were prepared from the same tissues and analysed by western blotting as described in Materials and Methods (C). The same cytosolic fractions were analysed for enzymatic activity by incubation with arachidonic acid and the products 12-HETE and 15-HETE were separated by reverse phase HPLC. The quantification was performed by automatic peak integration. The numerical values correspond to the production of µmol HETEs/250 mg tissue/15 min.

signal. Probes designed from genomic sequences up to 5 kb downstream of the 3'-end of 3'-UTR2 failed to hybridise specifically to reticulocyte poly(A)⁺ RNA in northern blots. Primer extension analysis gave no indication for multiple longer transcripts extending upstream to the 27 nt long 5'-UTR (not shown).

Tissue-specific expression of 15-LOX mRNAs differing in their 3'-UTRs

The existence of alternative transcripts of the *Alox15* gene raised the question of their functional significance. Therefore, the tissue specificity of their expression was investigated. Northern blots analysing poly(A)⁺ RNAs of 10 different tissues of rabbits made anaemic by bleeding (reticulocytes, bone marrow, leukocytes, brain, lung, trachea, gut, testis, bladder and heart) were hybridised with a 15-LOX coding probe (Fig. 2A), stripped and rehybridised with a 15-LOX 3'-UTR2 probe (Fig. 2B). It is evident that only the blood cells expressed the short 2.6 kb mRNA1, whereas all other tissues synthesised the long 3.6 kb mRNA2. In reticulocytes

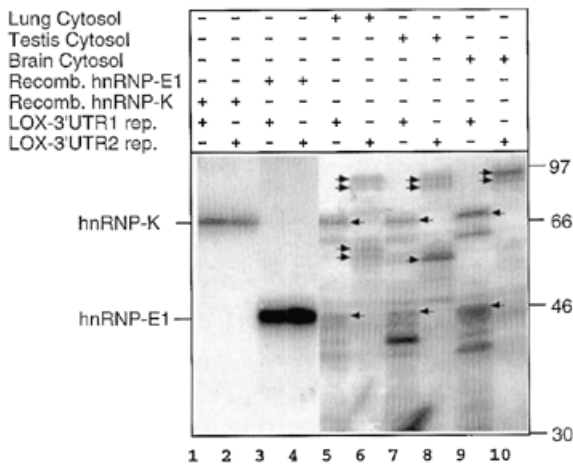


Figure 4. Binding of proteins to repetitive differentiation control elements (DICEs) in 3'-UTRs of 15-LOX mRNAs analysed by UV cross-linking. ³²P-labelled RNA transcripts representing rabbit 15-LOX 3'-UTR1 repeats (10R = DICE1) or 15-LOX 3'-UTR2 repeats (8R = DICE2) (Fig. 3) were incubated with recombinant rabbit hnRNP proteins E1 and K (lanes 1–4) or with cytosol of lung, testis or brain (lanes 5–10) as indicated and subjected to UV cross-linking and RNase digestion. The cross-linked proteins were analysed by 15% SDS-PAGE and autoradiography. The positions of proteins mentioned in the text are marked by arrows and marker proteins are indicated in kDa.

with His-tag modified human recombinant E1 and K proteins (24). To prevent any misinterpretation resulting from possible interspecies differences and the His tags, we decided to clone and express the homologous rabbit proteins without His tags which could interfere with RNA binding. Instead, the very efficient RNA affinity purification via biotinylated transcripts of the binding motif (DICE1) was used (23).

Clones for the hnRNP proteins E1 and K were isolated by screening a rabbit reticulocyte cDNA library in the vector λ ZAP Express (custom library; Stratagene) with human E1 and K cDNA probes. Full-length clones have been sequenced and the sequences have been deposited in the EMBL database under the accession nos AJ003023 and AJ003024. The deduced amino acid sequences of both proteins were identical to the corresponding human sequences except for Val205 of human E1 being replaced by Ala and Phe238 of human K being replaced by Tyr.

Recombinant rabbit hnRNP proteins E1 and K expressed in *E. coli* were then used in UV cross-linking experiments to study their binding to the DICE motifs residing in 3'-UTR1 and 2. As Figure 4 shows (lanes 1–4), recombinant proteins E1 and K bind equally well to 15-LOX DICE1 (10 repeats) and DICE2 (8 repeats). Similar results were obtained with the appropriate elements of the *Alox12* gene (DICE1 20 repeats and DICE2 9 repeats) (results not shown). In contrast, with cytosolic extracts from tissues known to express exclusively the long 3.6 kb 15-LOX mRNA (lung, testis and brain; Fig. 2) the DICE2 motif of 3'-UTR2 exhibited a completely different protein-binding pattern than the DICE1 motif of 3'-UTR1 (Fig. 4, lanes 5–10). From all three tissue extracts the DICE1 motif binds predominantly the hnRNP proteins E1 and K (marked with arrows in lanes 5, 7 and 9). In contrast to the results with purified recombinant E1 and K, the DICE2 motif failed to bind these proteins from a mixture of

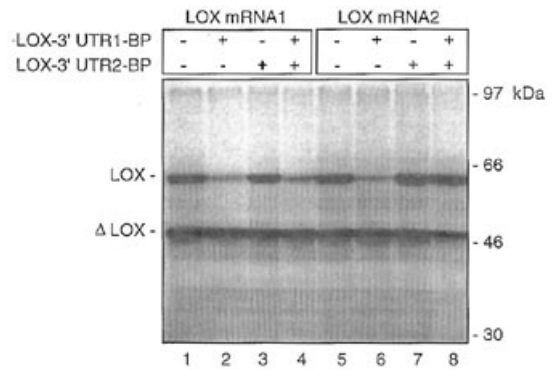


Figure 5. Translational inhibition of 15-LOX mRNAs 1 and 2 by 3'-UTR1 and 2 binding proteins. *In vitro* transcribed rabbit 15-LOX mRNAs 1 or 2 were co-translated with the 15-LOX transcript ΔLOX (deletion of 3'-UTR and amino acids 263–497 from 15-LOX mRNA1) in the presence of rabbit recombinant LOX 3'-UTR-binding protein hnRNP E1 (LOX 3'-UTR1-BP) or rabbit lung cytosolic protein affinity purified by binding to the 3'-UTR2 repeat of LOX mRNA2 (LOX 3'-UTR2-BP) in the rabbit reticulocyte lysate cell-free *in vitro* translation system in the presence of [³⁵S]methionine as described (22). Newly synthesised proteins were analysed by SDS-PAGE and autoradiography.

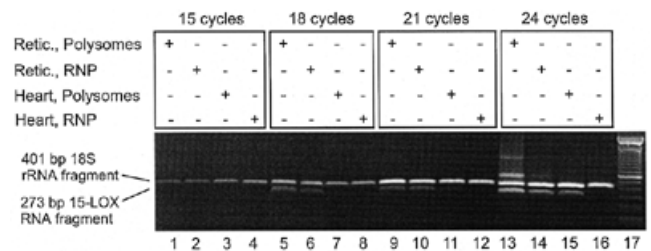


Figure 6. Quantification of 15-LOX mRNAs in polysomes and post-polysomal mRNPs by RT-PCR. Total RNA was isolated from rabbit reticulocyte and heart polysomes or post-polysomal RNP fractions as described in Materials and Methods. RT-PCR was performed for 15–24 cycles as indicated with 15-LOX-specific primers and with primers specific for 18S rRNA in the same reaction. DNA fragments were analysed by 1% agarose gel electrophoresis and visualised by ethidium bromide staining (lanes 1–17). Lane 17, DNA marker 100 bp ladder (Gibco BRL).

cytosolic proteins from cells known to express the 15-LOX2 mRNA. Instead, another set of polypeptides was bound migrating in the range 90–93 and 53–55 kDa (lanes 6, 8 and 10, see arrows).

Significance of 15-LOX 3'-UTR-binding proteins in translational control

To investigate their functional significance we tested the influence of recombinant hnRNP E1 and LOX 3'-UTR2-binding proteins purified by RNA affinity chromatography on the translation of 15-LOX mRNAs 1 and 2 by *in vitro* transcription/translation experiments (Fig. 5). It is evident that not only the reticulocyte 15-LOX mRNA1, but also 15-LOX mRNA2, which is restricted to non-erythroid tissues (lanes 2 and 6), is translationally repressed by recombinant hnRNP E1. An UTR2 repeat-binding

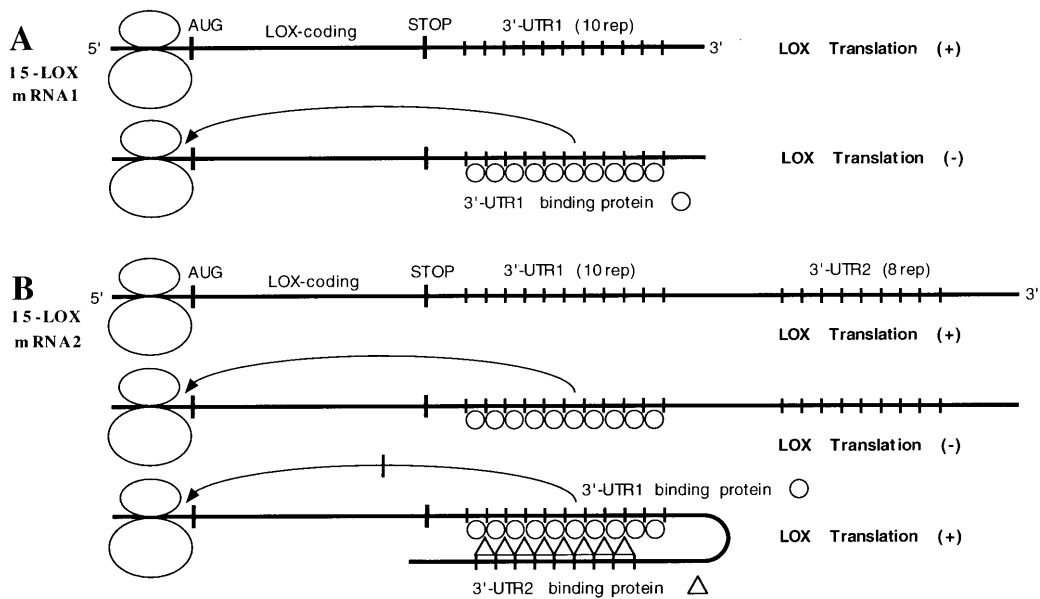


Figure 7. Model of translational control of 15-LOX synthesis by 3'-UTR-binding proteins. The rabbit 15-LOX mRNAs 1 (short 3'-UTR) (A) and 2 (long 3'-UTR) (B) are not drawn to scale.

protein fraction isolated from lung cytosol, which is mainly constituted of a polypeptide of ~93 kDa (lane 10), did not on its own influence the translation of either mRNA1 or mRNA2 (lanes 3 and 7). However, when it was added in combination with hnRNP E1 it prevented the translational inhibition of mRNA2, but not of mRNA1 (lanes 4 and 8). This indicates that UTR2-binding protein(s) antagonises the inhibitory effect of E1 but needs binding to the UTR2 repeat for its action. To exclude general effects on the translational efficiency by adding RNA-binding proteins co-transcription/translation has been performed with a LOX recombinant in which the 3'-UTR was deleted together with a small in-frame deletion in the coding region (Δ LOX) to allow its translation product to be distinguished from those of the LOX mRNAs 1 or 2. As can be seen (Fig. 5, lanes 2, 4 and 6), a LOX mRNA lacking its regulatory 3'-UTR is no longer susceptible to the inhibitory action of the 3'-UTR1-binding protein.

To further verify the finding that the long mRNA seems to exist in non-erythroid tissues in a translationally unrepressed state, we analysed the distribution of 15-LOX mRNAs between the polysomal (translationally active) and post-polysomal (translationally inactive) compartment of reticulocytes and heart by semi-quantitative RT-PCR. The results are shown in Figure 6. Only in reticulocytes are LOX-specific sequences, shown as a 273 bp PCR fragment, localised to a substantial amount in the post-polysomal RNP fraction (~50%), whereas in heart the long 15-LOX mRNA is found exclusively in polysomes (lanes 13–16; 24 cycles). Increasing the number of cycles up to 30 did not amplify LOX-specific sequences from heart RNP fractions. Repetition of the PCR reaction using 15-LOX2-specific primers designed from 3'-UTR2 revealed the same result with heart polysomes (not shown). Under identical conditions no LOX mRNA2-specific sequences can be amplified from reticulocytes, showing that the result obtained with this tissue is specific for

mRNA1. PCR was performed under quantitatively comparable non-saturating conditions. To demonstrate this the PCRs in which lower number of cycles were used are also shown (lanes 1–12). Quantification was normalised by comparing samples with equal amounts of rRNA, visualised as a 401 bp 18S rRNA PCR fragment.

DISCUSSION

The search for alternative transcripts of the *Alox15* gene led to the detection of a 15-LOX mRNA with an alternative 3'-UTR extending the main 2.6 kb mRNA by 1019 nt (Fig. 1). The functional significance of the longer alternative 15-LOX mRNA was addressed by determining the tissue distribution of 15-LOX mRNA1 and mRNA2 by northern blotting, immunoblotting and product analysis in a selection of rabbit tissues (Fig. 2), *in vitro* translation experiments (Fig. 5) and investigation of their intracellular distribution (Fig. 6).

The short 2.6 kb mRNA1 is the absolutely dominant form in reticulocytes, whereas heart, trachea, lung, bladder, testis, gut and brain express exclusively the 3.6 kb mRNA2. In bone marrow and blood leukocytes both mRNAs can be seen. However, it is difficult to decide whether these tissues synthesise both mRNAs or whether the mRNA1 signal originates from contaminating reticulocytes.

Recently, an alternative transcript of the human *Alox15* gene has been described which is very similar to the 3.6 kb rabbit 15-LOX mRNA2 (32). The rabbit 15-LOX mRNA2 contains an additional 3'-UTR sequence of similar length (1180 versus 1019 nt), however, with a rather different structure. The elements differ mainly in two features: (i) the presence of two Alu sequences in the human LOX 3'-UTR2, which are absent in the rabbit 3'-UTR2; (ii) the existence of the repetitive DICE motif in

the rabbit sequence which is absent in the human sequence. A 400 nt section of the 3'-UTR between the two Alu repeats is the only region to show significant homology (70%) to the corresponding part of the rabbit mRNA sequence, demonstrating their common evolutionary origin.

Analysing the genomic structure of the *Alox15* gene confirmed that UTR1 and UTR2 were not further interrupted by an intron but have different polyadenylation sequences. Many transcription units contain multiple polyadenylation sites, however, little is known about the rules that govern choices between them (reviewed in 33,34). Generally, alternative 3'-UTRs can be generated by alternative splicing or alternative polyadenylation. Human splicing factor PR264/SC35 is an example where both mechanisms operate (35). In this case, alternative 3'-UTRs result in different mRNA half-lives. Although alternative polyadenylation is a common occurrence, few examples are found in the literature showing tissue specificity in the choice between poly(A) sites located in a single terminal exon. Three such examples are the mRNAs encoding translation factors eIF-4E (36) and eIF-5 (37) and tyrosine hydroxylase of *Drosophila* (38). However, there is currently no information about the tissue-specific factors involved.

The leukocyte-type 12-LOX gene also carries elements of the DICE1 type (20 repeats) and of the DICE2 type (nine repeats) (Fig. 3). Although no functional mRNA2 of the 12-LOX mRNA has been found yet, it is obvious that the corresponding region of the 12-LOX gene (exon 14b, Fig. 3) does contain the same motif with nine subunits instead of eight in the case of the 15-LOX gene. Sequence analysis of the genomic region of the 12-LOX gene corresponding to exon 14b showed that the polyadenylation signal AATAAA of the 15-LOX gene was mutated to AATGAG. This could have caused a loss of functionality in polyadenylation and may explain why no 12-LOX cDNA containing a long 3'-UTR2 in analogy to 15-LOX cDNA2 has been detected. UV cross-linking patterns with *in vitro* transcripts of the putative control regions do not differ significantly from those obtained with 15-LOX DICEs (not shown). Currently it is unknown if the degree of repetitiveness plays a significant role in 12- and 15-LOX mRNA function.

It is generally accepted that the 3'-UTR and the length of the poly(A) tail are important determinants in the translational regulation of gene expression, particularly during embryonic development and differentiation (reviewed in 39,40). LOX mRNAs are an interesting subject for the investigation of the interaction of regulatory proteins with targets in the 3'-UTR of an mRNA. We could clearly demonstrate that hnRNP proteins E1 and K act as sequence-specific translational inhibitors by interaction with the DICE in the 3'-UTR of 15-LOX mRNA1 (24). The present study shows that recombinant rabbit hnRNP proteins E1 and K are able to bind equally well to both DICE1 and DICE2 motifs in 15/12-LOX mRNAs 1 and 2. If, however, a complex cytosolic extract is used as a source for E1 and K only DICE1 binds E1 and K, whereas DICE2 binds a different set of unidentified polypeptides in the range 53–55 and 90–93 kDa. hnRNP proteins E1 and K, as all K-domain proteins, are known for their oligo(C) binding capacity (29,30,41). So, it was not unexpected that the CU-rich DICE2 motif is able to bind E1 and K. However, in the cytosol of non-erythroid cells other proteins clearly compete very efficiently with E1 and K, preventing their binding. They could be part of the system necessary for the correct functioning of the long 15-LOX mRNA. Recently, the interaction of two proteins with a CU-rich element of 26 nt in the

3'-UTR of GAP-43 mRNA has been described (42). Interestingly, the 85 kDa protein FBP (far upstream element-binding protein) resembles hnRNP K and a 67 kDa protein shares sequence homology with PTB, a polypyrimidine tract-binding protein. The analysis of the novel DICE2-binding proteins will show if they belong to the K-domain family or to another family of RNA-binding proteins.

A first insight into a possible role of DICE2-binding proteins in non-erythroid cells came from translation inhibition experiments. From lung cytosol a polypeptide of ~93 kDa predominantly bound to the DICE2 of the 3'-UTR2 (Fig. 4). It was able to prevent repression of translation of LOX mRNA2 by hnRNP E1, not however of LOX mRNA1 (Fig. 5). Figure 7 describes schematically a scenario of how the components could interact. It is striking that both elements DICE1 and DICE2 have a repetitive structure. Earlier titration experiments, in which we studied E1 binding to DICE1, showed that the 10-fold repeat is able to bind up to 10 molecules of E1 or K (unpublished results). Obviously, the 93 kDa protein is only able to prevent translational inhibition when it is bound to DICE2, on LOX mRNA1 it is without any effect. This could be explained by a protein-protein interaction between multiple E1 and 93 kDa polypeptides which is dependent on binding of the 93 kDa proteins to DICE2. Such a spatial arrangement could block the interaction of DICE1/E1 with the 5' translation initiation complex, a necessary condition for the translational inhibition by hnRNP E1/K (23). This, however, has still to be shown experimentally.

Our experiments analysing the intracellular distribution of LOX mRNA1 in reticulocytes and LOX mRNA2 in heart (Fig. 6) support the view that LOX mRNA2 exists in non-erythroid tissues predominantly in a translationally non-repressed state caused by the action of 3'-UTR2-binding proteins. Whether the UTR2-binding proteins themselves are subjects of control circuits remains to be investigated.

ACKNOWLEDGEMENT

The authors wish to acknowledge support by the Deutsche Forschungsgemeinschaft to B.J.T. (grant Th 459/1-4).

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