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Cloning and Sequencing of the cDNA Encoding the Rubber Elongation Factor of *Hevea brasiliensis*

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ABSTRACT

In *Hevea brasiliensis,* the rubber particle in the laticiferous vessel is the site of rubber (*cis*-1-4-polyisoprene) biosynthesis. A 14 kilodalton protein, rubber elongation factor (REF), is associated with the rubber particle in a ratio of one REF to one rubber molecule (Dennis M, Henzel W, Bell J, Kohr W, Light D [1989] J Biol Chem 264:18618–18628; Dennis M, Light D [1989] J Biol Chem 264:18608–18617). To obtain more information concerning the function of REF and its synthesis and assembly in the rubber particle, we isolated cDNA clones encoding REF. We used antibodies to REF to screen a *Hevea* leaf λgt11 cDNA expression library and obtained several positive clones. Sequence analysis of the REF cDNA clones showed that the REF mRNA contains 121 nucleotides of 5′-nontranslated sequences and a 205 nucleotide 3′-nontranslated region. The open reading frame encodes the entire 14 kilodalton REF protein without any extra amino acids (Dennis M, Henzel W, Bell J, Kohr W, Light D [1989] J Biol Chem 264:18618–18628). The REF cDNA was subcloned in pGEM-3Z/-4Z and expressed *in vitro.* The translation product is a 14 kilodalton protein that can be immunoprecipitated with antibodies to REF. Addition of microsomal membranes to the *in vitro* translation product did not alter the mobility of the REF protein. This, and the sequence data, indicate that REF is not made as a preprotein. Our results suggest that REF is synthesized on free polysomes in the laticifer cytoplasm and that assembly of the rubber particles is likely to occur in the cytosol.

Rubber (*cis*-1–4-polyisoprene) is synthesized by over 1800 plant species distributed among 300 genera of seven families. Among these, the rubber tree, *Hevea brasiliensis,* is presently the sole commercial source of natural rubber. *Hevea* rubber is synthesized on spherical or pear-shaped particles of 50 Å-5 μm diameter. The RPs[2] are stored within the cytoplasm of specialized laticiferous cells, which anastomose and form a concentric network within the phloem tissue of the tree (9, 10, 13). Upon bark incision (tapping), the cytoplasm of the laticiferous vessels, which is referred to as latex, is expelled. Although rubber is the major component of *Hevea* latex, many other isoprenoids and their derivatives are also found (6, 11, 26).

Acetyl-CoA has been shown to be the precursor of isoprenoids and *Hevea* rubber (2, 3, 25). Condensation of three acetyl-CoA molecules, followed by reduction, phosphorylation, and decarboxylation results in the formation of IPP, which forms the basic unit for all isoprenoid biosynthesis. IPP isomerase (CD 5.3.3.2) converts IPP to dimethyl allyl pyrophosphate, which is the starter molecule for subsequent additions of IPP (24). In the biosynthesis of all isoprenoids, subsequent IPPs are added by prenyl transferases yielding C10, C15, etc. In the absence of a RP matrix, the *Hevea* latex prenyl transferase, rubber transferase (CD 2.5.1.20) (2), catalyzes IPP additions in a *trans*-configuration. However, when RPs are present, it catalyzes IPP additions in a *cis*-configuration to the rubber chains in the RP (17, 18). The amount of rubber transferase in *Hevea* latex saturates all available elongation sites, which account for up to 0.01% of the rubber molecules present in frozen and thawed whole latex samples (17, 18).

The most abundant protein of the RP is the REF, which plays a functional role in rubber polymerization (7, 8). Quantitative analysis of REF and rubber in whole latex reveals a ratio of one molecule of REF to one molecule of *cis*-1–4-polyisoprene (8). The protein can be easily purified as it remains attached to RP washed with 0.1% Triton X-100, which removes most other proteins associated with the RP (8). The primary structure of the REF protein, which has a M_r of 14,000, has been determined (7).

In this paper, we describe the isolation and sequencing of the full length REF cDNA clone. The nt sequence and *in vitro* expression data indicate that REF is not made as a preprotein. The implications of these findings for RP biogenesis are discussed.

MATERIALS AND METHODS

Screening of a *Hevea* Leaf cDNA Library

We obtained a λgt11 cDNA expression library prepared from leaf polyA RNA primed with oligo(dT) from Dr. M.-L. Chye at the Institute of Molecular and Cell Biology, Singapore. Rabbit antibodies to REF were prepared as described previously (8). The primary library (3 × 10^5 plaque-forming units) was plated on Y1090 cells and screened with anti-REF antibodies at 10 μg/mL according to the protocol described by Promega (21). Positive clones were purified and their *Eco*RI inserts subcloned in M13mp18/19 and sequenced using Bethesda Research Laboratories kilobase and New England Biolabs sequencing kits.

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[2] Abbreviations: RP, rubber particle; IPP, isopentenyl pyrophosphate; REF, rubber elongation factor; bp, base pair; nt, nucleotide.

Synthesis of Oligonucleotides

The oligonucleotides EG1 (23mer, GACGTTTGAGA-AGGTAGTCACAG) and EG3 (28mer, CCCTTGTTGGTT-GTCTTCGTCTTCAGCC) were synthesized using a Pharmacia oligonucleotide synthesizer and purified by PAGE (23).

RNA Extraction

Total RNA was extracted from latex using 2× extraction buffer according to the method of Prescott and Martin (20). PolyA RNA was purified by oligo(dT)-cellulose chromatography according to the method of Maniatis (19), except that LiCl was used instead of NaCl.

RNA Priming and Sequencing

Oligomers were labeled with [γ-^{32}P]ATP according to the method of Maniatis (19). The labeled oligomers EG1/3 (5.10^4 cpm) were annealed to 0.6 μg of polyA RNA at 60°C. Primer extension and sequencing were performed according to the method of Shelness and Williams (22) using the Bethesda Research Laboratories reverse transcriptase kit.

Screening of a *Hevea* Laticifer cDNA Library

The random-primed laticifer λgt10 cDNA library was a kind gift of Dr. V. Vanniasingham and S. Sivasubramaniam at the Institute of Molecular and Cell Biology, Singapore. The amplified library (4×10^5 plaque-forming units) was plated out on *Escherichia coli* C600 cells. Plaques were lifted onto Hybond-N nylon membranes (Amersham) and autoclaved to denature and fix the phage DNA (1). The oligonucleotide EG3 was labeled with [γ-^{32}P]ATP and the filters were hybridized with the probe overnight in 500 mM sodium phosphate (pH 7), 7% SDS, 15% formamide, and 1 mM EDTA at 60°C. The filters were washed twice for 5 min in 50 mM sodium phosphate (pH 7), 0.1% SDS at room temperature and twice for 5 min at 60°C. The washed filters were exposed for 12 h to 5 d at −70°C using intensifying screens (5). Positive plaques were purified and their *Eco*RI inserts cloned into pUC18. The resulting pUC18:LxEG3REF clones were subjected to restriction analysis (*Eco*RI/*Pst*I, *Eco*RI/*Eco*RV and *Pst*I) to determine the insert size and orientation.

λ-DNA Isolation

λ-DNA was isolated from plate washes using equilibrated DEAE cellulose (Whatman DE51, 100 mM Tris [pH 7.5]) to retain cell debris. After centrifugation, the supernatant was made 10 mM EDTA/0.1% SDS and incubated at 65°C for 20 min. After phenol:chloroform (1:1, v/v) extraction, the DNA was precipitated with 100 mM NaCl and 0.6 volume of isopropanol. The DNA was washed with 70% ethanol and used as such or further purified by CsCl gradient centrifugation.

Reconstruction of the Complete cDNA

The *Ssp*I/*Eco*RI fragment of pUC18:LfAbREF encompassing the 3′-end of the REF cDNA was altered by linker addition into a *Hind*III/*Eco*RI fragment and cloned into pGem-3Z/-4Z *Eco*RI/*Hind*III (21) to produce MS142.9/13. The 5′-end of the REF cDNA was cloned from pUC18:LxEG3REF as a 230 bp *Eco*RI/*Pst*I fragment into MS142.9/13 *Eco*RI/*Pst*I to produce pGEM-3Z/−4Z:REF.

In Vitro Transcription and Translation

pGEM-3Z/-4Z:REF (5 μg) was linearized with either *Eco*RI or *Hind*III. Transcription was carried out with T7 or Sp6 polymerase in the presence of 7-methyl-G cap (Pharmacia) according to the method of Promega (21). Sense or antisense

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cDNA                          GAATTCCGG
mRNA      NNNNNNNAAUCGGUUGAUAGCCUCCAUCAGCGTTTTCAGAAAGGCGGGTTTCTTTTTTGAAACTTAAGCGACTGCGTTT
             ,    ;    ,    ;    ,    ;    ,    ;    ,   50    ,    ;    ,    ;    ,

TGAATTTTGATCTTCCATTTTTGCAAAAGGAAATCTTCGATTATGGCTGAAGACGAAGACAACCAACAAGGGCAGGGGGAGGGGTTAAA
                                          M  A  E  D  E  D  N  Q  Q  G  Q  G  E  G  L  K
;    ,    ;    ,  100    ,    ;    ,    ;    ,    ;    ,    ;    ,  150    ,    ;    ,

                      ↓
ATATTTGGGTTTTGTGCAAGACGCGGCAACTTATGCTGTGACTACCTTCTCAAACGTCTATCTTTTTGCCAAAGACAAATCTGGTCCAC
 Y  L  G  F  V  Q  D  A  A  T  Y  A  V  T  T  F  S  N  V  Y  L  F  A  K  D  K  S  G  P  L
 ;    ,    ;    ,    ;    ,  200    ,    ;    ,    ;    ,    ;    ,    ;    ,  250    ,

TGCAGCCTGGTGTCGATATCATTGAGGGTCCGGTGAAGAACGTGGCTGTACCTCTCTATAATAGGTTCAGTTATATTCCCAATGGAGCT
  Q  P  G  V  D  I  I  E  G  P  V  K  N  V  A  V  P  L  Y  N  R  F  S  Y  I  P  N  G  A
  ;    ,    ;    ,    ;    ,    ;    ,  300    ,    ;    ,    ;    ,    ;    ,    ;    ,

CTCAAGTTTGTAGACAGCACGGTTGTTGCATCTGTCACTATTATAGATCGCTCTCTTCCCCCAATTGTCAAGGACGCATCTATCCAAGT
L  K  F  V  D  S  T  V  V  A  S  V  T  I  I  D  R  S  L  P  P  I  V  K  D  A  S  I  Q  V
 350    ,    ;    ,    ;    ,    ;    ,    ;    ,  400    ,    ;    ,    ;    ,    ;    ,

TGTTTCAGCAATTCGAGCTGCCCCAGAAGCTGCTCGTTCTCTGGCTTCTTCTTTGCCTGGGCAGACCAAGATACTTGCTAAGGTGTTTT
 V  S  A  I  R  A  A  P  E  A  A  R  S  L  A  S  S  L  P  G  Q  T  K  I  L  A  K  V  F  Y
    ;    ,  450    ,    ;    ,    ;    ,    ;    ,    ;    ,  500    ,    ;    ,    ;

ATGGAGAGAATTGAGCCCCAATTTGCACCAATAGCTTCCAACTAAGCAAGATAATGATATGCTCAAGAATATATATCTATTGTGAGCTT
  G  E  N  .
,    ;    ,    ;    ,  550    ,    ;    ,    ;    ,    ;    ,    ;    ,  600    ,    ;

TTTTTATGTTCTCATCCTGAGTGTTGAGACTATGTTTTCGTTTGAATATTAAACTGTGTTTTATTATGTGTTTTGAATATTCATAATGA

 ,    ;    ,    ;    ,    ;    ,  650    ,    ;    ,    ;    ,    ;    ,    ;    ,  700

GAATAAAGGGCCAATTGAATTATTGGCCAATATGTAATGATpA

  ,    ;    ,    ;    ,    ;    ,    ;743
```

Figure 1. Nucleotide sequence of REF cDNA and its derived amino acid sequence. The mRNA is comprised of 743 nt (without the polyA tail). The sequence of the first 29 nt was determined by RNA primer extension sequencing, whereas the remainder was determined from cDNA sequencing. The sequences complementary to the oligomers EG1 (204–226) and EG3 (124–151) are underlined. The 5′- and 3′-untranslated regions are 121 and 205 nt in length, respectively. The 5′-end of LfAbREF1 to 3 is indicated by a vertical arrow. The *Pst*I, *Eco*RV, and *Ssp*I sites are at 261, 272, and 660, respectively. The predicted amino acid sequence is given in single letter code.

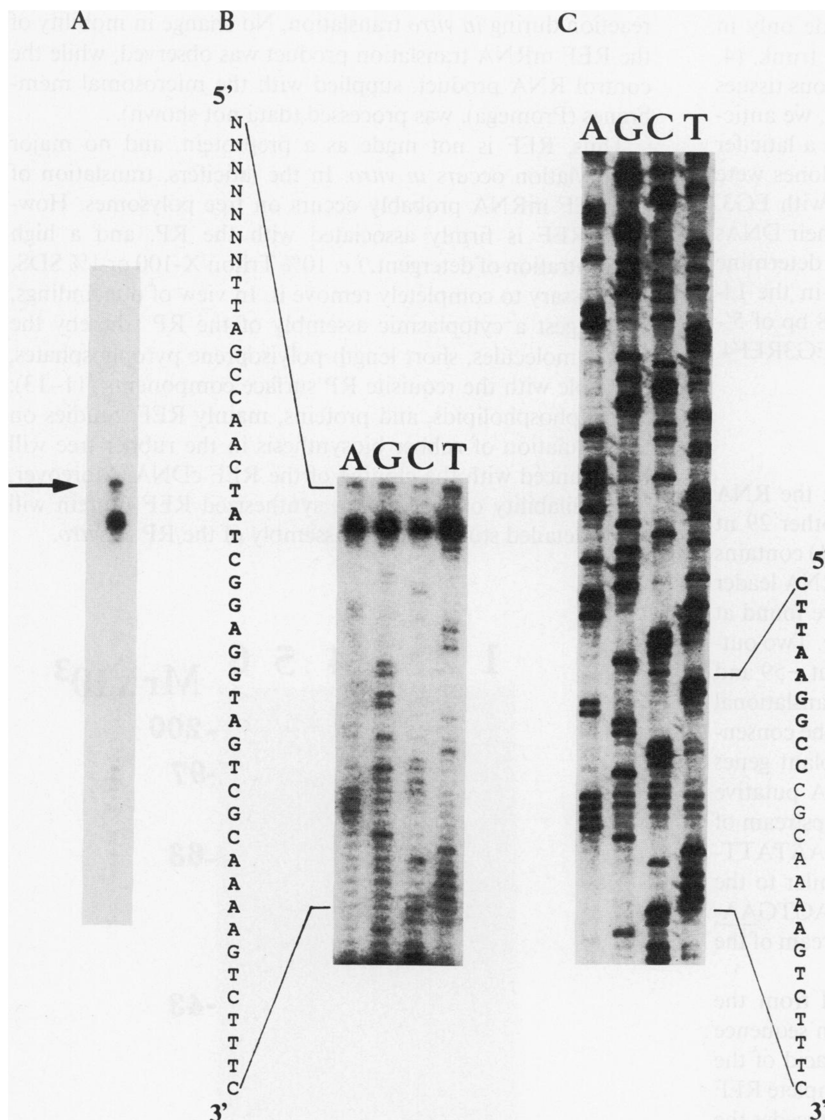

Figure 2. Mapping of the 5′-end of the REF RNA. A, RNA primer extension using EG3 as primer. Arrow denotes the 151 nt extended product. Its size was determined by comparison with the sequencing lanes in C. RNA (B) and cDNA (C) sequencing using EG3 as primer. Sequencing lanes were loaded from left to right in the order AGCT. The *Eco*RI linker in the cDNA is shown in bold type. N = nt not determined.

REF RNA (100 ng) and 1 μg of laticifer polyA RNA were translated *in vitro* in the presence of L-[^{35}S]methionine (Amersham) using Promega rabbit reticulocyte lysate. The translation product (10^6 cpm) was precipitated with anti-REF antibodies using protein A-Sepharose (Sigma). The immunoprecipitated proteins were separated by 15% SDS-PAGE (16) and electroblotted onto a Genescreen membrane (New England Nuclear, Dupont). The membrane was sprayed with Enhance Spray (New England Nuclear, Dupont) and exposed at −70°C using intensifying screens (5) for 16 h to 5 d.

RESULTS AND DISCUSSION

Isolation of the Complete REF cDNA

Antibody screening of the *Hevea* leaf cDNA library resulted in the isolation of three partial cDNA clones (LfAbRef1–3). Sequence analysis showed that these clones were independent. They all contained 564 bp followed by varying lengths of polyA (Fig. 1). Comparison of the deduced amino acid sequence encoded by the cDNA clones with the amino acid sequence of REF (7) showed that the cDNA sequences started at amino acid codon 27 and encoded the remainder of REF without any mismatches.

Although an additional 14 clones were isolated by further screening the cDNA libraries with ^{32}P-labeled REF probe, none of them extended the 564 bp partial cDNA clone at the 5′-end. We therefore determined the upstream sequences of the REF RNA by RNA primer extension and sequencing. As primer we used EG1, a 23mer homologous to nt 7 to 29 at the 5′-end of the LfAbREF cDNA clones. RNA primer extension and sequencing showed a strong stop, probably due to pausing of the avian myeloblastosis virus reverse transcriptase at a secondary structure in the REF RNA, 6 nt away from the EG1 primer. This may explain why the 5′-ends of several independent cDNA clones are identical. RNA sequencing extended the known REF sequence by almost 100 nt (data not shown). On the basis of the extended sequence, we designed another oligomer EG3, which was used to screen

a laticifer cDNA library. In *Hevea*, rubber is made only in the laticifers, which are mainly located in the tree trunk. (4, 9). Leaves contain only a small amount of laticiferous tissues (10). Based on the distribution of the REF protein, we anticipated REF cDNA to be much more abundant in a laticifer than in a leaf library. More than 1000 positive clones were obtained by screening the laticifer cDNA library with EG3. Six clones were purified (LxEG3REF1 to 6) and their DNAs digested with *Eco*RI/*Pst*I and *Eco*RI/*Eco*RV to determine which clones possessed 5′-sequences not present in the LfAbREF clone. We found that clone 4 contains 168 bp of 5′-sequences not found in LfAbREF. This clone, LxEG3REF4, was used to reconstitute the complete cDNA.

5′-Transcript Mapping and Sequencing

5′-Transcript mapping using EG3 revealed that the RNA extends beyond the complete cDNA clone by another 29 nt (Fig. 2, A and C). The complete REF mRNA (Fig. 1) contains a 5′-untranslated region of 121 nt. Within this mRNA leader two in-frame stop codons, "TGA" and "TAA," are found at −42 and −57 relative to the first ATG, respectively. Two out-of-frame stop codons T′GA and TG′A are found at −59 and −35, respectively. The sequence surrounding the translational start site "TCGATTATGGCT" is homologous to the consensus sequence "TAAACAATGGCT" derived for plant genes (14). The 3′-untranslated region is 205 nt long. A putative polyadenylation signal AAUAAA is found 39 nt upstream of the polyA tail. Again, the context of this signal "GAATATTCATAATGAGAATAAAGGGCCAATTG" is similar to the consensus sequence "TATAAT/AT/AAAAAAAGTGAATAAAAA/GA/TTAT/AAAT/AT" at 27 ± 9 upstream of the polyadenylation site (15).

The amino acid sequence of REF as deduced from the cDNA sequence is identical to the primary protein sequence determined by Dennis *et al.* (7). The first amino acid of the protein is acetylated alanine. Sequencing of the complete REF cDNA clone shows that the initiator methionine precedes the N-terminal alanine, which is ultimately modified. The absence of a signal sequence was further demonstrated by 5′-sequencing and transcript mapping of the REF mRNA. Thus, posttranslational modification of REF is minimally a two step process: the removal of the N-terminal methionine and the acetylation of the newly exposed alanine.

In Vitro Transcription and Translation

To investigate other potential modifications in an *in vitro* system, the complete cDNA clone was subcloned into pGEM-3Z/-4Z and transcribed *in vitro* in both directions from the Sp6 and T7 polymerase promoters in the vector. After *in vitro* translation, only the sense RNAs produced a translation product that was recognized by antibodies to REF. The size of the translation product was identical to the anti-REF immunoprecipitated translation product of the laticifer polyA RNA (Fig. 3). The antibodies to REF also immunoprecipitated a 58 kD latex protein, which is a translation product of the laticifer RNA (Fig. 3, lane 2, upper band). To investigate any cotranslational processing of the *in vitro* translation product, dog pancreas microsomal membranes were added to the reaction during *in vitro* translation. No change in mobility of the REF mRNA translation product was observed, while the control RNA product, supplied with the microsomal membranes (Promega), was processed (data not shown).

Thus, REF is not made as a preprotein, and no major glycosylation occurs *in vitro*. In the laticifers, translation of the REF mRNA probably occurs on free polysomes. However, REF is firmly associated with the RP, and a high concentration of detergent, *i.e.* 10% Triton X-100 or 1% SDS, is necessary to completely remove it. In view of our findings, we suggest a cytoplasmic assembly of the RP whereby the starter molecules, short length polyisoprene pyrophosphates, assemble with the requisite RP surface components (11–13): lipids, phospholipids, and proteins, mainly REF. Studies on the regulation of rubber biosynthesis in the rubber tree will be advanced with the cloning of the REF cDNA. Moreover, the availability of the *in vitro* synthesized REF protein will allow detailed studies on the assembly of the RP *in vitro*.

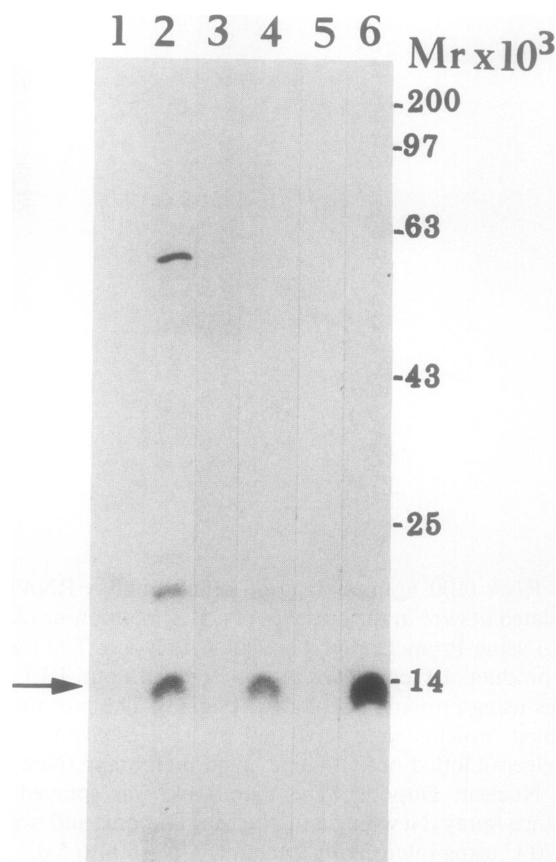

Figure 3. Analysis of *in vitro* translation products. After *in vitro* translation, samples were immunoprecipitated with antibodies to REF and separated on a 15% SDS-PAGE gel. The arrow denotes the 14 kD REF product. 1, no RNA; 2, 1 μg of latex polyA RNA; 3, 0.1 μg of antisense REF RNA transcribed from pGEM-3Z:REF *Eco*RI using Sp6 polymerase; 4, 0.1 μg of sense REF RNA transcribed from pGEM-3Z:REF *Hind*III using T7 polymerase; 5, 0.1 μg of antisense REF RNA transcribed from pGEM-4Z:REF *Eco*RI using T7 polymerase; 6, 0.1 μg of sense REF RNA transcribed from pGEM-4Z:REF *Hind*III using Sp6 polymerase.

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