First Apyrase Splice Variants Have Different Enzymatic Properties*

Received for publication, February 15, 2000, and in revised form, April 15, 2000
Published, JBC Papers in Press, April 12, 2000, DOI 10.1074/jbc.M001245200

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LALP70 is a novel lysosomal membrane protein belonging to the apyrase protein family. The apyrase protein family comprises enzymes capable of cleaving nucleotidetri- and diphosphates in a calcium-magnesium-dependent manner, not being altered by P-type, F-type, or V-type NTPase inhibitors. In this study we have cloned and sequenced the human LALP70 gene to determine the genomic structure. The gene is organized in 11 introns and 12 exons covering a genomic region of approximately 16 kilobase pairs. By fluorescence in situ hybridization analysis, the hLALP70 gene was mapped to the human chromosome 8p21.1-p21.3. We further show that there is at least one alternatively spliced variant, hLALP70v, which can be generated via an alternative splice side at the 3'-end of exon 7, leading to a protein variant differing in 8 amino acids (VSFASSQQ). This is the first splice variant that has been described in the apyrase protein family. Reverse transcriptase polymerase chain reaction analysis showed an ubiquitous expression of both variants, with different relative mRNA expression levels in different tissues. Comparison of the enzymatic properties of the splice variants revealed a broader substrate specificity for hLALP70v with respect to ATP, UTP, GDP, and GDP as preferred substrates, while hLALP70 utilized UTP and TTP preferentially. Furthermore, enzyme activity of hLALP70v was equally dependent on Ca$\textsuperscript{2+}$ and Mg$\textsuperscript{2+}$, being saturated already at 1 mM concentration. In contrast, hLALP70 enzymatic activity was unsaturated up to 10 mM Ca$\textsuperscript{2+}$, while Mg$\textsuperscript{2+}$ showed a saturation at already 1 mM concentration with 2-3-fold lower enzymatic activity as observed with Ca$\textsuperscript{2+}$. Our data suggest that the presence or absence of the 8-amino-acid motif VSFASSQQ provokes differences in substrate specificity and divalent cation dependence of hLALP70/hLALP70v.

The apyrase protein family comprises enzymes capable of cleaving nucleotidetri- and diphosphates in a calcium- or magnesium-dependent manner, not being altered by P-type, F-type, or V-type NTPase inhibitors (1). Members of the apyrase family contain up to four homolog conserved sequence stretches, which have been described as apyrase conserved regions (ACRs); Ref. 2. The substrate specificity differs between different apyrases and seems to be sensitive to small changes in the amino acid sequence outside the ACRs. This was shown for the 97% identical apyrase isoforms (NTP1 and NTP3) of Toxoplasma gondii (3, 4) and in human brain E-type apyrase after side-directed mutagenesis of two conserved tryptophan residues (5). Apyrases with a substrate specificity restricted to nucleotide triphosphates are classified as E-type ATPases (1, 6). Originally, apyrases have been described as ectoenzymes, like CD 39 (7), or other ecto NTPases (1, 6, 8). However, meanwhile several apyrases have been localized intracellularly (2, 3, 9–11).

The human lysosomal apyrase-like protein (hLALP70) belongs to the apyrase gene family, based on four ACRs in the N-terminal half of the sequence (11). The hLALP70 cDNA has an open reading frame of 1848 bp, encoding a protein with a molecular mass of 71 kDa (11). Based on sequence analysis, hydrophobicity plot and in vitro transcription/translation studies, hLALP70 is thought to be inserted in the transmembrane as a type III membrane protein (11, 12), with one transmembrane domain at the C terminus and two transmembrane domains at the N terminus of the protein. The amino acid sequence of hLALP70 is nearly identical with the sequence of the human UDPase (hUDPase; Ref. 10), except for an additional stretch of 8 amino acids, located 7 amino acids downstream from the ACR4 in the hLALP70 protein. We have isolated and analyzed the hLALP70 gene and provide evidence that the hUDPase is a splice variant of hLALP70. This is the first splice variant described so far for the apyrase family. RT-PCR analysis revealed a differential expression of hLALP70v and its splice variant, hLALP70. Finally we show that the two proteins differing in the 8 amino acids exhibit differences in their enzymatic properties.

EXPERIMENTAL PROCEDURES

Cloning and Analyzing of the Human LALP70 Gene—For isolation of genomic clones containing the human LALP70 gene, high density PAC hybridization filters (RLDB2, Berlin, Germany) were used. As probe a 321-bp fragment of the hLALP70 gene, representing the 3'-portion of the cDNA sequence (GenBankTM accession number AJ131258), was amplified using primers (LALP70, 5'-CCG GCC GTT TTC GCC ATC CAT TTT GTC TAC-3' and LALP1r, 5'-CTC GCC ATC CAT TTG TTC TTT-3'), annealing temperature: 58 °C). After hybridization a strong signal for PAC131C11, and weaker signals for several additional clones, were observed.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AJ246165.

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1 The abbreviations used are: ACR, apyrase conserved region; DAPI, 4,6-diamino-2-phenylindole; hLALP70, human lysosomal apyrase-like protein of 70 kDa; mLALP, mouse lysosomal apyrase-like protein; hLALP70v, human variant of lysosomal apyrase-like protein of 70 kDa; mLALPv, mouse variant lysosomal apyrase-like protein; NTPase, phosphotriphosphatidase; NTP, triphosphate nucleotide; NDP, diphosphonucleotide; RT-PCR, reverse transcriptase polymerase chain reaction; FISH, fluorescence in situ hybridization; EST, expressed sequence tag; GFP, green fluorescence protein; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; bp, base pair(s); FITC, fluorescein isothiocyanate; kb, kilobase pair(s).

19018 This paper is available on line at http://www.jbc.org

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observed. PAC131C11 was used for the determination of the genomic structure by sequencing.

Sequencing templates were generated by means of PCR using cDNA-derived primers. PCR products were separated by gel electrophoresis and purified using the QiAQuick PCR Purification Kit (Qiagen). Approximately 75 ng of purified DNA was used for cycle sequencing. The cycle sequencing was performed according to manufacturer’s recommendations using ABI Prism BigDye terminator kit (PE Biosystems). Products from cycle sequencing were concentrated using standard ethanol precipitation procedure and analyzed on a ABI 373 automated sequencer (Applied Biosystems). The resulting sequences were aligned by the software package Sequencer (Gene Codes, Ann Arbor, MI).

To confirm that LALP70 and LALP70v (see below) are transcripts from the same gene, we performed a PCR analysis using primers LALP20r and LALP30r (Fig. 3A; LALP20f, 5′-ATGGGCCGCTGTCGACT-3′; LALP20r, 5′-TTAGAGGGAGTCTGGTCCT-3′; LALP30f, 5′-TGTATTTCAACACTGACG-3′; LALP30r, 5′-AGTCTCAATCTCGAGTCCTC-3′; annealing temperature 55 °C). As template we used either genomic DNA obtained from six unrelated individuals or the PAC131C11 DNA, respectively. Southern blot analysis was performed according to standard protocols. As hybridization probe we used a 400-bp PCR product generated with the LALP20r primers from the LALP70 cDNA as template. LALP20r primers are identical to those used by West and Guidotti (10).

Chromosomal Localization—The chromosomal localization of human LALP70 gene by fluorescence in situ hybridization (FISH) analysis was carried out on spreads of human lymphocyte metaphase chromosomes, with biotinylated PAC131C11 DNA as probe as described previously (13). In brief, 30 ng of total PAC DNA was labeled with biotin-7-dATP by nick translation (Nick Translation Kit, Life Technologies, Inc.) and detected after hybridization procedure with FITC streptavidin, biotinylated goat anti-streptavidin, and FITC streptavidin (Vector Laboratories, Burlingame, CA). Chromosome counterstaining was performed using DAPI, and slides were embedded in Vestashield (Vector). The preparations were analyzed with a Zeiss epifluorescence microscope, equipped with a CCD camera (Photometrics, München, Germany) controlled by the software package Smartcapture (Vysis, Bergisch-Gladbach, Germany).

Radiation Hybrid Panel Screen—Additional to FISH localization, hLALP70 was mapped with the Genebridge 4 radiation hybrid panel consisting 93 radiation hybrid clones of the human genome (Research Genetics). Screen were performed in duplicate, and the results of the PCR analysis were submitted to the server at the Whitehead Institute/MIT Center for Genome Research’s radiation hybrid map of the human genome. RT-PCR Analysis—For the isolation of total RNA samples from different organs indicated in Fig. 3 were snap-frozen in liquid nitrogen and stored at –80 °C. Tissue samples were extracted with RNAclean (AGS) according to the manufacturer’s instructions. 6 μg were used for the RT reaction in a total volume of 20 μl. As primer the lower primer indicated in Fig. 3 (20 pmol). Reverse transcription was performed for 2 h at 42 °C with 10 units of a reverse transcriptase from avian myeloblastosis virus (U. S. Biochemical Corp.). 5 μl from the RT reaction mixture was used for a subsequent PCR in a total volume of 50 μl using 20 pmol of each primer indicated in Fig. 3 and 10 units of Taq polymerase (Promega). The annealing temperature was set to 56 °C and the PCR was run for 35 cycles. 20 μl of the PCR reaction mixture were analyzed on a 3% Metaphor-agarose gel (FMC Bioproducts) in TBE buffer (90 mm Tris base, 10 m m boric acid, 2 mm EDTA).

Cloning of Expression Vectors—The full-length hLALP70 cDNA plus 105 bp 5′- and approximately 350 bp 3′-untranslated regions was subcloned into the pMCS-5 vector (MoBiTec, Göttingen, Germany) using FseI and XhoI restriction sites after a complete digest with FseI and a partial digest with XhoI. The hLALP70 cDNA was generated from the hLALP70-pMCS plasmid by deletion of the 24 bp between nucleotides 1028–1051 by PCR amplification using the Quick Change Site-Directed Mutagenesis kit from Stratagene. The sense deletion primer 5′-GCG TAC GAA GTC CCC AAA ACT GAA GTA GTC C-3′ was complementary to the antisense deletion primer 5′-AGC TTC TCT TTC AGT TTT GGC GAC TTC GTA CGC-3′. The deletion mutagenesis was performed according to the manufacturer’s instructions. The obtained cDNA was provided in Escherichia coli XL1-blue supercompetent cells (Stratagene). The correctness of the 24-bp deletion was confirmed by DNA sequencing. Both hLALP70 and hLALP70v were cloned into the mammalian expression vector pCL-Neo (Promega) using MluI and XbaI restriction sites.

Cell Culture and Transfection—COS-7 cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 2 g/liter Hepes, 5% fetal calf serum, 5% adult calf serum, 50 μg/ml gentamycin, and incubated at 37 °C in a humidified chamber equilibrated with 5% CO2. COS-7 cells were transfected with the mammalian pCL-Neo expression vector alone or with this vector containing either the cDNA for hLALP70 or for hLALP70v, respectively, using a 25-μl polyethyleneimine (Aldrith) as transfection reagent. Transfection procedure was performed as described elsewhere (Biederbeck et al. (11)). Transfected cells were harvested for the apyrase enzymatic assay 24 h after transfection.

Preparation of COS Cell Crude Membranes—Transfected COS-7 cells were homogenized with a Dounce homogenizer, and nuclei were separated as described elsewhere (14). To separate the crude membrane fraction from the cytosol the postnuclear supernatant were centrifuged at 105,000 × g. The pellets were resuspended in 400 μl of 20 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.2 mM EDTA containing 0.1% Triton X-100. The protein concentration in each sample was determined with bicinchoninic acid (Sigma) according to the manufacture’s instructions.

Measurement of Nucleotide Phosphatase Activity—To measure apyrase activity COS-7 membrane suspension containing 6 μg total protein were adjusted to 45 μl with reaction buffer containing 20 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.2 mM EDTA, 1 mM Mn2+ and 0.5 mM Na2VO3, with or without 5 mM CaCl2. After preincubation of 5 min at 25 °C the nucleotide phosphatase reactions were initiated by the addition of 5 μl of the same buffer containing 10 μM nucleotide phosphate substrates to give a final concentration of 1 μM. Samples were incubated for 20 min at 37 °C. NTP/NDP hydrolysis under these conditions were linear up to 30 min. Apyrase activity was determined by measuring the inorganic phosphate released as described previously (15, 16). Values obtained from samples without CaCl2 or MgCl2 were subtracted from those obtained with CaCl2 or MgCl2. All measurements were done in triplicate.

RESULTS

Cloning, Sequencing, and Mapping of the hLALP70 Gene—Primers specific for the human LALP70 gene identified a PAC clone (131C11) with a 80-kb insert (data not shown). This PAC clone was subsequently used for primer-directed sequence walk to analyze the genomic structure of the human LALP70 gene. Our sequence analysis of the hLALP70 gene revealed that it contains 12 exons and 11 introns, covering a genomic region of approximately 16 kb (Fig. 1A; Table I). The sizes of the exons range from 8 nucleotides (exon 1) to 325 bp (exon 9). Sequence analysis of the exon-intron junctions revealed that all splice sites obeyed the GT-AG paradigm (Table I). The hLALP70 gene containing PAC131C11 clone was found to hybridize specifically at a single locus on the short arm of chromosome 8 (Fig. 2A), and no specific signals were observed on any other chromosome. DAPI banding analysis indicated the localization of hLALP70 to the chromosomal region 8p21.1-p21.3 (Fig. 2B). To map the gene more precisely, we used for radiation hybrid mapping the Genbridge G4 panel (Research Genetics, Huntsville, AL) and PCR. Primers for mapping were described above. We determined that the gene is located 0.4 centiRay distal to WI-861 (LOD >3.1), which is located in the Genethon map between the markers D8S1734 and D8S1820 (44.9 and 54.2 centiMorgan from the top of chromosome 8, respectively). In this area the EST sequence KIAA0392, which represents the 3′-end of the hLALP70v cDNA (GenBank Accession number AF106002), has been located previously (17).

hLALP70 is almost identical to a Golgi apyrase (10) except for an additional 24-base pair insert in the hLALP70 sequence. We localized these 24 bp at the end of exon 7. The 5′-end of the 24-bp stretch provided an alternative splice site (Fig. 1B). Based on this, we concluded that the human Golgi apyrase might be a splice variant of hLALP70, further designated as hLALP70v. In the paper published by Wang and Guidotti (10) the authors used a genomic Southern blot approach with EcoRI or NdeI-digested genomic DNA and a 400-bp fragment from the hUDPase cDNA as hybridization probe, which did not contain a EcoRI or NdeI restriction site. Since they obtained
two bands they speculated that there might be two similar genes. Correlation of the 400-bp fragment to the LALP70 gene structure showed that the sequence contained the entire exon 8, as well as parts of exon 7 and exon 9 (Fig. 3A). To confirm that hLALP70 and hLALP70v are transcripts from the same gene that comprises about 16 kb and consists of 12 exons. The transmembrane domains and the ACRs I–IV are indicated. The size of introns and exons are shown in Table I. The corresponding GenBank™ accession number is AJ246165. B, part of exon 7 illustrating the alternative splice sites used to generate either hLALP70 or hLALP70v, respectively.

Identification and Expression of a mLALP Splice Variant—In subsequent experiments we analyzed the expression of mLALP and mLALPv in 14 different mouse tissues by a RT-PCR approach (Fig. 4A). Primers were designed according to a mouse EST (GenBank™ accession number AA497420) representing the hLALP70 mouse homolog (mLALP) containing the 24 bp (Fig. 4A). PCR primer were flanking the 24 bp. The 5′-sequence of the upper primer was complemented with a sequence from the human LALP70, since only 11 bp of the 5′-upstream mLALP sequence were available from the data base (Fig. 4A). Results from the RT-PCR approach revealed that both mLALP and mLALPv were expressed in all tissues (Fig. 4B). However, the relative level of mLALPv was always much lower than that of mLALP. The highest expression of mLALPv were found in liver and kidney (Fig. 4B). We conclude that mLALP and mLALPv are both ubiquitously expressed, but that the levels of expression might be tissue-specific.

Analysis of Substrate Specificity—In our next experiment we analyzed the enzyme substrate specificity of the human LALP70 and its splice variant hLALP70v to determine the influence of the 8 amino acids VSFASSQQ on the enzyme activity. Data from other apyrases have shown that only slight differences in the sequence can have a marked influence on the enzyme activity. Data from other apyrases have shown that only slight differences in the sequence can have a marked influence on the enzyme activity.
substrate specificity (data not shown). For this reason we cloned hLALP70 and hLALP70v into an expression vector without any tag. Using lysates from cells that had been transfected with these constructs we measured the apyrase activity for a variety of NTPs and NDPs as indicated in Fig. 5. While hLALP70 exhibited the highest enzyme activity on UTP and TTP, hLALP70v cleaved CTP most efficiently followed by CDP, UDP, and GTP. Thus, hLALP70v has a broad substrate specificity indicative for a typical apyrase based on a definition by enzymatic properties (1). According to these results, hLALP70v does not represent a typical UDPase as has been published on the basis of enzyme data obtained with a LALP70v/myc fusion protein (10).

Analysis of Divalent Ion Dependence—Since apyrase activity usually depends on the presence of divalent cations, we compared hLALP70 and hLALP70v activity under a concentration range of Ca$^{2+}$ and Mg$^{2+}$, respectively. As substrate we used UTP for hLALP70 and CTP for hLALP70v. hLALP70 apyrase activity was already saturated at 1 mM Mg$^{2+}$ (Fig. 6A). At 5 mM concentrations the enzyme activity was three to four times higher with Ca$^{2+}$ than with Mg$^{2+}$. With hLALP70v, a rapid increase of enzyme activity was observed up to 1 mM Ca$^{2+}$ and Mg$^{2+}$, comparable with the kinetic observed for hLALP70v (Fig. 6B). However, total enzyme activity of hLALP70v was 2–3-fold higher than of hLALP70 for both Ca$^{2+}$ and Mg$^{2+}$. Enzyme activity was saturated for Ca$^{2+}$ and Mg$^{2+}$ concentrations.
mLALPv. mLALP, the 131-bp band represents a cation. While the 155-bp band represents total RNA from mouse organs as indicated. While the 155-bp band represents total RNA from mouse organs as indicated. While the 155-bp band represents total RNA from mouse organs as indicated.

that the first 8 nucleotides of the upper primer were derived from the human sequence. This indicates that the fragments observed are due to splicing differences in the human and mouse sequences. The 400-bp fragment, which did not contain a Nde I site, was obtained with Ca²⁺ or Mg²⁺ as described under “Experimental Procedures.” Different NTP and NDP substrates were used as indicated. The assay was performed in the presence or absence of 5 mM Ca²⁺, and the data obtained with Ca²⁺ were corrected with the values obtained without Ca²⁺. Mean values from three separate measurements are given.

higher than 1 mM (Fig. 6B). Thus, hLALP70 can use either Ca²⁺ or Mg²⁺ equally, while LALP70 shows a preference for Ca²⁺.

A crude membrane preparation from cells transfected either with a hLALP cDNA, or a hLALPv cDNA, or the vector alone was used to measure the nucleoside phosphatase activity in the presence of Na₃P and Na₃VO₄ as described under “Experimental Procedures.” Different NTP and NDP substrates were used as indicated. The assay was performed in the presence or absence of 5 mM Ca²⁺, and the data obtained with Ca²⁺ were corrected with the values obtained without Ca²⁺. Mean values from three separate measurements are given.

DISCUSSION

The human apyrase LALP70 is identical to the human Golgi UDPase (10), with the exception of additional 24 bp, which are removed in the splice variant hLALP70v. Short bold sequences represent the upper and lower primer, respectively, which were used for RT-PCR. Note that the first 8 nucleotides of the upper primer were derived from the human sequence. B, RT-PCR results obtained with total RNA from mouse organs as indicated. While the 155-bp band represents mLALP, the 131-bp band represents mLALPv.

In preliminary RT-PCR experiments using primer flanking the 24-bp sequence we observed a differential expression of hLALP70 and hLALP70v in different human cell lines (data not shown). To investigate the expression pattern of mLALP and mLALPv in different tissues, we designed PCR primer flanking the 24 bp stretch in the mouse EST (Fig. 4A). Since only a few bases 5’-upstream of the 24-bp sequence were given 9 flanking the 24 bp stretch in the mouse EST (Fig. 4A). Since only a few bases 5’-upstream of the 24-bp sequence were given.

Thus, it cannot be excluded that in some cell types, which contribute only to a small percentage to a given tissue, the mLALP/mLALPv ratio is inverted. Moreover, the turnover rates of the two proteins might be different adjusting the protein amount of mLALP and mLALPv in the cell to other levels as reflected by their relative amount of RNA. While our data clearly show that both mLALP and mLALPv are expressed ubiquitously, the detailed and cell-specific expression levels of these two proteins have to be elucidated in further experiments on RNA and protein level.

In initial experiments to measure the hLALP70 enzyme activity we used hLALP70 and a hLALP70/green fluorescent protein (GFP) fusion protein. We observed that the GFP had an influence on the substrate specificity (data not shown). Thus, in
cies on minor sequence differences. Apyrase isoforms (NTP1 and NTP3) encoded from two different genes in *Toxoplasma gondii* had an amino acid sequence identity of 97% but differed markedly in their substrate binding and specificity (4). This was attributed to two 12-amino acid-long sequences, FITGREMLASID and IVTGGGLMAA1N, which are located at the C terminus of these proteins, where they constitute the region of highest dissimilarity between the two isoforms. However, no similarity between our 8-amino acid sequence and the two 12-amino acid sequences are obvious. Furthermore, a human brain ectoapyrase was used for site-directed mutagenesis of two conserved tryptophan residues (W187A, W459A; Ref. 5), which are also conserved in hLALP70 (Trp227, Trp526; Ref. 11). While the W187A mutation abrogated the enzymatic activity, a stimulation of the NTPase activity was observed in case of the W459A mutation. Other single amino acid mutations revealed similar results (6). Finally, in addition to the changes in substrate specificity, the hLALP70-specific motif VSFASSQQ has also an influence on the enzymatic dependence on divalent ions like Ca^{2+} and Mg^{2+}.

hLALP70v, which has been described as a Golgi-resident protein (10), is a homolog to the yeast intracellular apyrase GDA1, also located in the Golgi apparatus. GDA1 functions as a GDPase, converting GDP to GMP, which is then transported from the Golgi lumen into the cytosol with a GDP activated sugar (9). Since GDP-activated sugars are essential for protein glycosylation in the Golgi compartment, GDA1 mutants exhibit less glycosylated proteins and an increased level of GDP in the Golgi cisternae (9). Recently, a mouse UDPase has been cloned and characterized, which is localized in the endoplasmic reticulum (ER-UDPase; Ref. 19). It is thought that the ER-UDPase can unfold a similar function in the ER as GDA1 in the Golgi complex, being critical in the reutilization of UDP during reglycosylation of misfolded glycoproteins (20–22). ER-UDPase, which sequence is unrelated to that of the hLALP proteins, is a soluble protein. In contrast to hLALP70v and to the ER-UDPase, hLALP70 has been located in autophagic/lysosomal vacuoles (11). It has to be elucidated by further experiments, whether hLALP70 is also active in the reutilization of NTPs/NDPs from the lysosomal/autophagic compartment, as has been proposed earlier (11). In lysosomes only nucleoside transport systems have been described so far (23), and one can assume that NTPs and NDPs trapped in a lysosomal vacuoles have to be degraded at least to NMPs, in analogy to the Golgi compartment and the rough endoplasmic reticulum (RER), to be transported into the cytosol. However, since the lysosomal compartment does not provide a protein glycosylation machinery, it is unlikely that the outward transport of monophosphate nucleotides or nucleosides is linked to an in-
ward transport of NDP-activated sugars, as it has been de-
scribed for the Golgi compartment (9) and the RER (21, 22).

Although most of a transiently expressed hLALP70/GFP fu-
sion protein was colocalized with lamp1-positive vacuoles, a
further association with the Golgi apparatus and the ER could
not be ruled out (11). Furthermore, the Golgi localization of
hLALP70v has been shown only indirectly (10), and it remains
to be shown that this splice variant is exclusively located in this
compartment or might also occur in lysosomes. Thus, the ques-
tion remains open whether the amino acid motif VSFASSQQ
also contains sorting information.

Acknowledgments—We thank Melanie Hudler and Hartmut Engel
for expert technical assistance and Ralf Roesser for preparing the
electronic file of the manuscript.

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