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Structure of the Chromosomal Gene and cDNAs Coding for Lactase-Phlorizin Hydrolase in Humans with Adult-Type Hypolactasia or Persistence of Lactase

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Summary

Lactase-phlorizin hydrolase (LPH) splits lactose in the small intestine. LPH activity is high in the suckling; in many human populations the activity declines in adults, leading to adult-type hypolactasia, whereas in other populations the high LPH activity persists in adults. In the present work, we compared LPH sequences at the gene and cDNA level among adult subjects with high and low LPH activity. The complete intron-exon organization, including the sequences of all 17 exons and of the borders of all introns (as well as about 1,000 bp of 5′ flanking region), was established for the cloned chromosomal LPH gene of a subject with persistence of lactase. Using PCR, we directly sequenced the exons of a hypolactasic subject. Except for silent mutations and the unknown linkage phase at two heterozygous positions, both coding sequences were identical. We further examined the LPH mRNA of a hypolactasic subject by S_1 mapping and by sequencing a set of overlapping PCR products produced from cDNA templates. Except for allelic differences, the LPH sequence of the hypolactasic subject was identical to that of the LPH cDNAs of three subjects with persistence of lactase (one cDNA isolated previously by cloning and two characterized in the present work by PCR). No allele was peculiar to the hypolactasic subject. We conclude that humans with high or low levels of lactase can code for identical LPH enzymes.

Introduction

Lactase-Phlorizin Hydrolase (LPH)

In the mammalian small intestine, lactase-phlorizin hydrolase (LPH) digests lactose, the sugar of milk, to glucose and galactose. LPH is a "stalked" intrinsic protein of the brush-border membrane: the active portion of the enzyme protrudes into the lumen of the intestine, and is anchored to the membrane by a hydrophobic segment near the carboxy terminus (Mantei et al. 1988). LPH has two catalytic activities, lactase proper and an aryl- or alkyl-β-glycosidase ("phlorizin hydrolase"). The enzyme is synthesized as a large precursor of molecular weight >200,000 (Danielsen et al. 1984; Skovbjerg et al. 1984; Büller et al. 1987; Naim et al. 1987), which is processed to give the final LPH of molecular weight about 135,000–160,000 (Danielsen et al. 1984; Büller et al. 1987; Naim et al. 1987). Biochemical data and cDNA cloning (Mantei et al. 1988) have established that pre-pro-LPH comprises five domains: (1) a putative signal sequence of 19 amino acids, (2) a large pro-region of 849 amino acids (in human), none of which is found in mature LPH in the brush border membrane, (3) an extracellular domain of 1,014 amino acids, carrying both active sites of the enzymes, (4) a stretch of 19 hydrophobic amino acids serving as a membrane anchor, and (5) a short cytoplasmic segment of 26 amino acids at the carboxy terminus. The cDNA was found to be fourfold internally repeated. Two repeats are in the pro-region; the two further repeats in mature LPH very likely correspond to lactase and phlorizin hydrolase domains.

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Developmental Regulation of LPH

LPH activity is relatively high during the suckling period, but in most human populations, as well as in other mammals, the specific activity (units per gram of mucosal protein) later declines some 3–20-fold, leading to decreased ability to digest lactose ("adult-type hypolactasia" or "lactose intolerance"; Auricchio et al. 1963; Dahlqvist et al. 1963). Note that this condition is distinct from human congenital absence of LPH, which is very rare. Genetic evidence (reviewed in Flatz 1989) indicates that primary adult-type hypolactasia in humans is an autosomal recessive trait, and that persistence of LPH activity (lactose tolerance) in adult life is dominant. High frequencies of persistence of lactase activity are found, in the Old World, primarily in northern Europe and among certain populations with a tradition of dairying in Northern Africa and Arabia. Hypolactasia is the predominant phenotype among the native populations of Australia and Oceania, East and Southeast Asia, tropical Africa, and the Americas. Intermediate frequencies are found in, for example, southern Europe. For reviews on adult-type hypolactasia see Kretchmer (1971) and Semenza and Auricchio (1989). The LPH gene has been mapped to the long arm of chromosome 2 (Kruse et al. 1988, 1989), but it is not yet known whether lactase persistence and nonpersistence are linked to this locus.

What is the basis for the developmental switch in LPH activity, and why does this switch not operate in the case of lactase persistence? Using cDNAs for rabbit and human LPH (Mantei et al. 1988), and for rat LPH (N. Mantei, unpublished data), we have recently examined LPH RNA levels during development (Sebastio et al. 1989): in rabbit and rat, the RNA is high in both suckling animals and in adults. Concordant conclusions have been reached by Freund et al. (1989, 1990); conflicting results for rat were reported by Büller et al. (1990). In the case of humans (Sebastio et al. 1989; subjects from Naples were studied), hypolactasic subjects showed on the average 50% as much LPH RNA as subjects with persistence of lactase. This decrease was clearly far less marked than the 10–20-fold drop in enzyme activity. Also, at least one hypolactasic subject showed a high level of LPH RNA, comparable to those in subjects with persistence of lactase. We concluded that in our Neapolitan study population mRNA accumulation is not the main site of control in adult-type hypolactasia, and that some form of posttranscriptional control is likely.

The enzymes found in adults with low and high lactase levels appear identical by various biochemical and physical criteria, including electrophoretic mobility (Crane et al. 1976) as well as immunological properties and specific enzymatic activity (Lorenz-Meyer et al. 1972; Skovbjerg et al. 1980; Potter et al. 1985). However, subtle differences may have gone undetected. In the present work, we established the complete intron-exon organization, including the sequences of all exons and the flanking regions, for the cloned chromosomal LPH gene of a subject with persistence of lactase. Using PCR, we then directly sequenced the exons of a hypolactasic subject. Except for silent mutations, the sequences of both subjects were identical. Further, examination of LPH cDNAs from subjects with high and low lactase demonstrated that there was no alternative splicing or RNA editing. We conclude that the primary pre-pro-LPH translation products have identical sequences in subjects with persistence of lactase and hypolactasia, and that therefore any posttranslational regulatory mechanism must operate on identical pre-pro-lactase species.

Material and Methods

Experimental Subjects

We compare sequence data from four subjects with persistence of lactase (P1–P4) and two with adult-type hypolactasia (H1 and H2). P1 is an organ donor from Zurich, from whose intestinal RNA the original LPH cDNA clone was isolated (Mantei et al. 1988). P2 is of northern European ethnic background by way of the United States. The other subjects are from Naples. Table 1 summarizes the available data on lactase and sucrase activities, as well as LPH mRNA levels, in intestinal mucosa homogenates from these subjects. P2 was shown to be lactose tolerant by measurement of blood glucose after ingestion of lactose (methods reviewed by Semenza and Auricchio [1989]). Subjects P1, P4, and H2 have been described before (Sebastio et al. 1989).

Isolation and Analysis of Genomic Clones

Blood DNA prepared (Herrmann and Frischauf 1987) from subject P2 was partially digested with *Sau*3aI and fragments of 15–23-kb isolated by agarose gel electrophoresis. These were cloned into the λDASH vector (Stratagene), packaged (Gigapack Gold, Stratagene), and screened without amplification with human LPH cDNA excised from plasmid pHLac61 (Mantei et al. 1988). Overlapping clones were found by hybridizing each insert with dot blots of all the others. Restrictions maps were prepared by

Table 1

Disaccharidase Activities and LPH mRNA in Intestinal Mucosa

Subject	Lactase (U enzyme/g mucosal protein)	Sucrase (U enzyme/g mucosal protien)	Sucrase/Lactase	LPH mRNA (pg LPH mRNA/μg total RNA)
P1.....	15[a]	52[a]	3.5	2.4
P3.....	30[a]	56[a]	1.9	172
P4.....	87	251	2.9	24
H1.....	6	125	21	ND
H2.....	10[a]	464[a]	46	17

NOTE.—Lactase and sucrase activities were determined in homogenates of jejunal mucosa (Sebastio et al. 1989). Less than 13 U lactase/g protein accompanied by a ratio of sucrase to lactase activities above 4.1 is considered diagnostic for hypolactasia (Welsh et al. 1978; Flatz 1989). LPH mRNA was determined by an S_1-mapping procedure.

[a] Data from Sebastio et al. (1989).

ND = not determined.

analyzing total and partial (Smith and Birnstiel 1976; Rackwitz et al. 1984) digests of phage DNA.

Southern blots of phage and genomic DNA were prepared using a vacuum blotting apparatus (Pharmacia-LKB) to transfer DNA to Zeta-Probe membranes (Bio-Rad) and hybridized with labeled (Feinberg and Vogelstein 1983) cloned LPH DNA.

Fragments subcloned into M13mp19 (Norrander et al. 1983) were sequenced using the dideoxy method (Sanger et al. 1977) with a T7 sequencing kit from Pharmacia. Sequencing gels were read with the aid of a Graf-Bar sonic digitizer (Scientific Accessories) and a program written for the Macintosh computer. The sequences were assembled and analyzed with the Genetics Computer Group program package (Devereux et al. 1984) on a VAX 8700.

PCR Amplification and Sequencing

Primers of length 19 to 22 nucleotides were synthesized on a Pharmacia Gene Assembler. PCR was performed with 1 μg genomic DNA prepared as above, or with cDNA derived from 2 μg total RNA. The cDNA was prepared (Tung et al. 1989) by reverse transcription of intestinal mucosa RNA (isolated by a lithium chloride–urea method [Auffray and Rougeon 1980]), using oligo $(dT)_{12-18}$ or random hexanucleotides (Pharmacia) as primer. cDNA primed with random hexanucleotides gave a considerably lower background in the PCR.

Genomic DNA was amplified by 30 cycles of PCR (1 min at 95°C, 2 min at 55°C, and 2 min at 72°C). cDNA was amplified with 35–40 cycles (53°C hybridization), the product isolated by agarose gel electrophoresis, and 2–10 ng amplified with a further 20–30 cycles (50°C hybridization). Final products were isolated by gel electrophoresis, and about 0.05–0.1 pmol subjected to 10 cycles of "single-stranded PCR" (Kreitman and Landweber 1989). The single-stranded product was extracted with phenol/chloroform (1:1) and unincorporated nucleotide triphosphates removed by two cycles of isopropanol precipitation from 2.5 M NH_4Ac. This material was sequenced with a T7 DNA polymerase sequencing kit (Pharmacia), following the supplier's protocol except that the "labeling mix" was diluted threefold and only 5 μCi ^{35}S-dATP per reaction were used.

The PCR and sequencing primers are listed in table 2.

S_1 Mapping and Primer Extension Analysis

S_1 mapping was carried out with end-labeled probes (Weaver and Weissmann 1979) prepared from various LPH clones (genomic DNA, cDNA, and hybrids of the two), or synthesized using single-stranded PCR (Kreitman and Landweber 1989) on an LPH cDNA template with an end-labeled oligonucleotide as primer. Primer extension was according to the method of Boorstein and Craig (1989) using an end-labeled oligonucleotide (table 2, no. 70) as primer.

Quantitative S_1 mapping was carried out as described by Sebastio et al. (1989). Some samples described in that paper were reassayed for the present work.

Results

Isolation of the Chromosomal LPH Gene

Blood DNA from a subject with persistence of lactase (P2) was partially digested with *Sau*3aI and cloned in the λDASH vector (Stratagene). About 1.8×10^6 plaques were screened with human LPH cDNA (from

Table 2

Oligonucleotides used in PCR, Sequencing, and Primer Extension Analysis

Name	Length	PCR	Sequencing	Sequence	Position
1. HLACEX1AP	22	+	+	GCAGTTATAA AGTAAGGGTT CC	F EX 1
2. HLACEX1AM	20	+	+	TCTTGAGGGC CTTGAGGAGT	375
3. HLACEX1BP	20	+	+	ATTTCTGTCA TGGGCACAGC	278
4. HLACEX1BM	19	+	+	GTGTGTGATG AAGGTTGCC	F EX 1
5. HLACEX2P	20	+	+	CAGTGGTTTC CACAGTCAGA	F EX 2
6. HLACEX2M	20	+	+	CTCTCCTCAG ATGTTACAGG	F EX 2
7. HLACEX3P	21	+	+	TGCTTAGGTT CTAATGACCC T	F EX 3
8. HLACEX3M	21	+	+	CCTTCGCTGC ATTCATCATT A	F EX 3
9. HLACEX4P	20	+	+	AGATGCTTTC CAGGAGGCTT	F EX 4
10. HLACEX4M	20	+	+	GTGCACCAGA TGTCACTCAA	F EX 4
11. HLACEX5P	21	+	+	CTTGCATGTT CTTAGTTGGC A	F EX 5
12. HLACEX5M	21	+	+	CCCAAGAGTT GATGACAACA G	F EX 5
13. HLACEX6AP	20	+	+	ATGTCCCTTT TTGTCCCTTC	F EX 6
14. HLACEX6AM	20	+	+	CCAGGAGATG GAGAACTTGT	1382
15. HLACEX6BP	20	+	+	CTCTGACGTC GCCCTGCTTT	1322
16. HLACEX6BM	20	+	+	CCAATGTCCT TTCTCCTCCA	F EX 6
17. HLACEX7AP	22	+	+	GTTCATTAGT CCTGTTGTTG TC	F EX 7
18. HLACEX7AM	20	+	+	TTCTCTGCCT CTGTGAACTC	2023
19. HLACEX7BP	20	+	+	CTGAGGACCC AGATCCAACA	1944
20. HLACEX7BM	20	+	+	CACTGGCACA TCCATTGTTC	F EX 7
21. HLACEX8AP	21	+	+	CACAGCTATC AAGGAAGACT C	F EX 8
22. HLACEX8AM	20	+	+	TTCACATTGC TCCCTGGTGT	2842
23. HLACEX8BP	20	+	+	CGTGGGATGC CGATGGCAAA	2773
24. HLACEX8BM	20	+	+	CTGAGCCATA ACCTAGCCAT	3240
25. HLACEX8CP	21	+	+	CAGACCTTTG GTGATAGAGT C	3162
26. HLACEX8CM	20	+	+	AGTAGTACGT GTTGAGGCAG	3642
27. HLACEX8DP	20	+	+	TGCCAAGCTT CACTGAGGAA	3568
28. HLACEX8DM	20	+	+	CCCAGGGAAT GTTGGAAGAT	F EX 8
29. HLACEX8ZP	26	+	+	TAAATAAATC TAATTTATTT CTTCAC	F EX 8
30. HLACEX8SEQ1M	17		+	AGTAGGCAGA TTTCCTG	2520
31. HLACEX9P	20	+	+	TCCTGGTGTC AAGCTCTCCT	F EX 9
32. HLACEX9M	20	+	+	ACAGGGCAGG TGTGCAGTCA	F EX 9
33. HLACEX10P	20	+	+	GCTGTGAGTG CTTGACTGAA	F EX 10
34. HLACEX10M	20	+	+	TTCCTGCATG TGGACTTTCC	F EX 10
35. HLACEX11P	18	+	+	GATCTGGCTT CTTCCCAG	F EX 11
36. HLACEX11M	20	+	+	CTTGATGTGC TAAGAATGCC	F EX 11
37. HLACEX12P	22	+	+	CTCAGTCACA TCCATTCTAT CC	F EX 12
38. HLACEX12M	23	+	+	CACAATCTGT TTCCATTAGG CTG	F EX 12
39. HLACEX13P	20	+	+	GATGCTGTCT CTTGCTGTGC	F EX 13
40. HLACEX13M	21	+	+	TTGCCCGAGA CATGCCTCTG T	F EX 13
41. HLACEX14P	20	+	+	CTTCTCAAAG CATGGCTCTC	F EX 14
42. HLACEX14M	20	+	+	CCTTTTCCAG AGATGGCTCC	F EX 14
43. HLACEX15P	20	+	+	GTGTTTCGTG TAAAGGGCCT	F EX 15
44. HLACEX15M	20	+	+	AGCAGATGTT TCCAACAGGG	F EX 15
45. HLACEX16P	22	+	+	GCTGCCTTAT GAGATAGGAA AC	F EX 16
46. HLACEX16M	20	+	+	TTTATTCCCT CCCAGAGCCA	F EX 16
47. HLACEX17P	22	+	+	GATCTGAGAA CTCAAATCAG CG	F EX 17
48. HLACEX17M	20	+	+	TCAGCAGAGT CTAAGACCCT	5887
49. HLACEX17BP	20	+	+	GAGTTACCAC CTCAAGTTCT	5797
50. HLACEX17BM	20	+	+	AGCTTAATCG GAGCTCCAGA	F EX 17
51. HLACEX17SEQ1M	17		+	CTCCCTTAAC AACCCTT	6133
52. HLACCDNA1P	20	+		GTTCCTAGAA AATGGAGCTG	1
53. HLACCDNA2P	20		+	TCTCTGTTGT CCTGCGAGCT	661
54. HLACCDNA3M	17		+	TTTTAGGTTG AAGATGA	857

(*continued*)

Table 2

Name	Length	PCR	Sequencing	Sequence	Position
55. HLACCDNA4P	17		+	CTGCCTATCA GAGAGTC	1081
56. HLACCDNA5P	17		+	GTGACCTTCC ATGAGCC	1623
57. HLACCDNA6M	17		+	TTCCAGGGAT ACAAACT	2237
58. HLACCDNA7M	20	+	+	TGAGCCTCAC GTAGTAGTTC	4440
59. HLACCDNA8P	20	+	+	TCACCCTGCA GAACCTGGGT	4321
60. HLACCDNA9P	17		+	CCTACATTGT TGGCCAC	4702
61. HLACCDNA10P.....	17		+	TCACTACACC ACTGTCC	5054
62. HLACCDNA11P.....	20	+		GTCTGCATTT TGTGAACTAC	5437
63. HLACCDNA12M	20	+		CCACAGAGGC GTAGAACTTC	5514
64. HLACCDNA13M	21	+		TTTTGAAAGG ACAGTCTTCT G	6278
65. HLACCDNA14M	17		+	ACCAAGTGAG CCACCTT	1742
66. WCHRLAC1P	17		+	TTTGGACTGT ACCATGT	4005
67. WCHRLAC2M.....	17		+	CCGTTGTTGG TAATGAC	4099
68. WCHRLAC3M.....	17		+	ACCTGAACAT ATCTCCT	F EX 12
69. WCHRLAC4M.....	17		+	ACAGTGGTGT AGTGATT	5068
70. HLACEXTPRIM1M....	20			TATCAGACTC CCAGTCTGAC	87

NOTE.—Names ending in P are plus strand, those ending in M are minus strand. Plus signs under "PCR" and "Sequencing" indicate whether the oligonucleotides were used for PCR, for DNA sequencing, or for both. In the last column, primers within the coding region are indicated by numbers which give the position of the 5′-most nucleotide of the primer in the cDNA sequence (Mantei et al. 1988), and primers complementary to intron sequences are designated "F EX" followed by a number indicating the exon which the primer flanks.

subject P1) (Mantei et al. 1988), yielding 16 positive phages. Overlapping clones were identified by hybridization, and their inserts analyzed by restriction mapping and Southern blotting. The LPH gene is about 55 kb in length and is contained in the six clones shown in figure 1 (During culture of clone 26, deletions arose in the segment spanning the gap between clones 7 and 15; the size of the gap was confirmed by Southern blot analysis of genomic DNA). All clones formed a single linkage group.

The restriction map shown in figure 1 accounts for all the bands seen in a Southern blot when, for example, *Bam*HI-digested genomic DNA was probed with LPH cDNA (fig. 2). A probe derived from exon 1 gave band intensities with cloned and genomic DNA which were consistent with a single-copy gene (data not shown). Southern blot analysis of genomic DNA from a subject with persistence of lactase (P2) and a hypolactasic subject (H1) yielded identical band patterns (fig. 2). We conclude there is one LPH gene, which is similarly organized in the two genotypes.

Intron-Exon Organization

The intron-exon organization of the LPH gene was

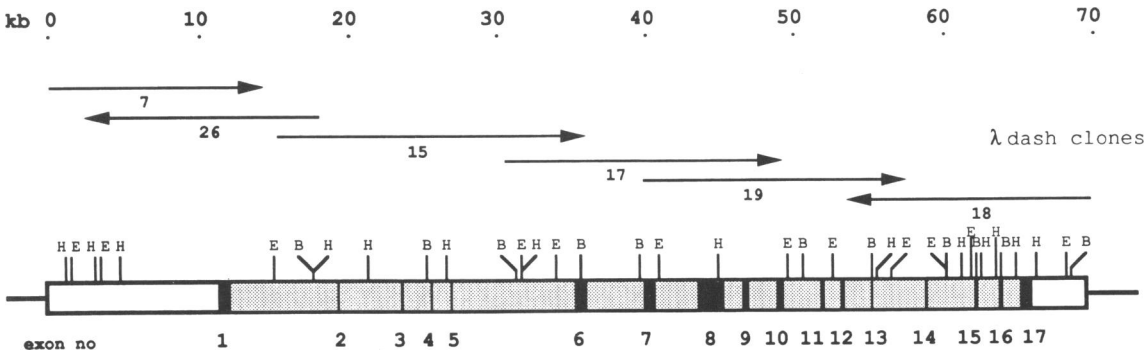

Figure 1 Structure of human LPH gene. The top line shows a scale in kilobases. The set of λDASH clones which covers the gene is indicated by the arrows; to indicate the orientation of the inserts within the vector, the arrowheads point to the right in the conventional λ map. In the restriction map, black boxes represent exons (exon numbers shown below), stippled boxes introns, and open boxes flanking sequences. Various restriction sites are indicated (H = *Hin*dIII; E = *Eco*RI; B = *Bam*HI).

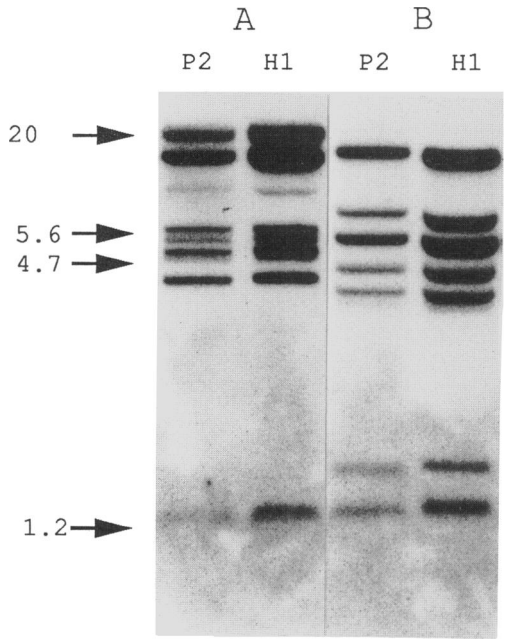

Figure 2 Southern blot analysis of LPH gene. DNA from subjects P2 (7.5 μg) and H1 (20 μg) was digested with *Bam*HI (lanes A) or *Bam*HI plus *Hin*dIII (lanes B), electrophoresed in a 1% agarose gel, blotted, hybridized with labeled LPH cDNA (clone pHlac61; Mantei et al. 1988), and washed in 1 × SSPE at 65°C. Sizes of marker fragments in kilobases are on the left.

determined by sequencing those segments of the gene containing exons. These were located in two steps: lambda clones 7, 15, 17, 18, and 19 (fig. 1) were cleaved with *Bam*HI, *Hin*dIII, *Eco*RI, or combinations of these enzymes. Fragments containing exons were identified by Southern blotting with LPH cDNA as probe. These fragments were isolated on a preparative scale, digested with the frequent cutter *Sau*3aI, and fragments with exons again located by Southern blotting. Positive fragments were subcloned in M13 vectors and sequenced.

The LPH gene proved to be divided into 17 exons (figs. 1, 3*B*), ranging in size from 79 to 1,551 bp. The intron-exon boundaries (fig. 3*B*) all fit well to the splice-site consensus sequences of Mount (1982). (Further data on intron sequences have been submitted to the EMBL/GenBank/DDBJ nucleotide sequence databases.)

Cap Site and Promoter Region

When the 5′ end of LPH RNA was analyzed in an S_1-mapping procedure (Weaver and Weissmann 1979), the protected fragments given by natural LPH mRNA (from both hypolactasic subjects and subjects with persistence of lactase) and an in vitro transcript from cloned cDNA (plasmid pHlac61; Mantei et al. 1988) were of identical length (Sebastio et al. 1989; and W. Boll, unpublished data), suggesting that the end of the mRNA lies at or within a few nucleotides of the 5′ end of the cDNA sequence (nucleotide 1026 in fig. 3*A*). This assignment was further investigated in a primer extension analysis. A primer complementary to nucleotides 87–68 of the cDNA sequence was hybridized to intestinal mucosa RNA from subjects P3 and P4 and elongated with reverse transcriptase. The major products ended at nucleotides 1021–1025 of the sequence shown in figure 3*A*, with only minor amounts of longer material (fig. 4). Note that with P3 there is a more or less even distribution of products among four ends, whereas with P4 a single major product (ending at nucleotide 1025) is seen. Considering the primer extension and S_1-mapping data together, and the fact that mRNAs always or almost always begin with purine nucleotides (Baralle and Brownlee 1978), we conclude the 5′ ends (or end) of LPH RNA lie(s) between nucleotides 1022 and 1026.

A typical TATA element (TATAAA, nucleotides 994–999) lies 23–27 nucleotides upstream of nucleotide 1022–1026, consistent with a site of transcription initiation near the latter positions.

The sequence of a further 1,000 nucleotides of 5′-flanking region is presented in figure 3*A*.

Coding Regions

The coding region of P2's LPH gene was found to be identical to that reported previously (Mantei et al. 1988) for a cDNA (subject P1) except for two silent mutations and three conservative replacement mutations (table 3 compares P1 and both alleles of P2; see below).

We then wished to compare these sequences with those from hypolactasic subjects, with the expectation that consistent differences in predicted amino acid sequence between the two groups would provide a clue to possible differences in protein processing or stability. We took advantage of PCR (Saiki et al. 1988) to rapidly sequence the coding region of the LPH gene of a hypolactasic subject (H1). The shorter exons were amplified using pairs of primers complementary to the flanking introns; for longer exons we also used internal primers and amplified overlapping segments of the exons. PCR products were isolated by gel electrophoresis, asymmetrically amplified with one primer (Kreitman and Landweber 1989) to provide single-stranded

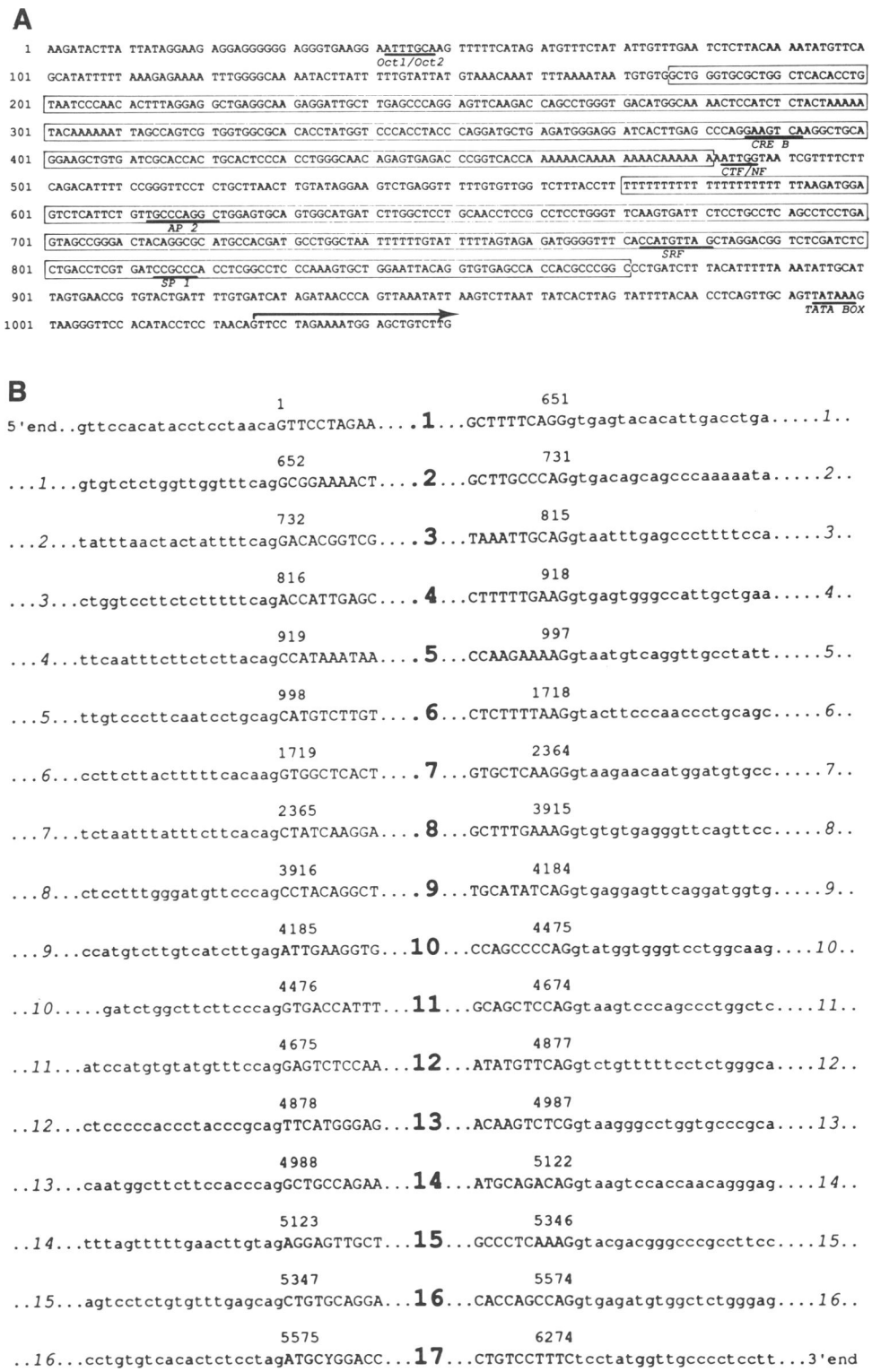

Figure 3 Sequence of 5′-flanking region and of intron-exon boundaries of LPH gene. *A,* Sequence of 5′-flanking region of the LPH gene carried in clone λhchrlac7 (see fig. 3). Potential promoter elements are underlined and *Alu* sequences are boxed. The start of the LPH cDNA found in clone pHlac61 (Mantei et al. 1988) is indicated by the arrow. *B,* Intron-exon boundaries of LPH gene. Fragments of chromosomal LPH clones (fig. 1) containing exons were subcloned and sequenced to determine the intron-exon organization. The exons are numbered in bold type, the introns in italics. The positions of the first and last nucleotides of the exons relative to the cDNA sequence (Mantei et al. 1988) are given above the sequences. Exon sequences are in capital letters, intron sequences in small letters.

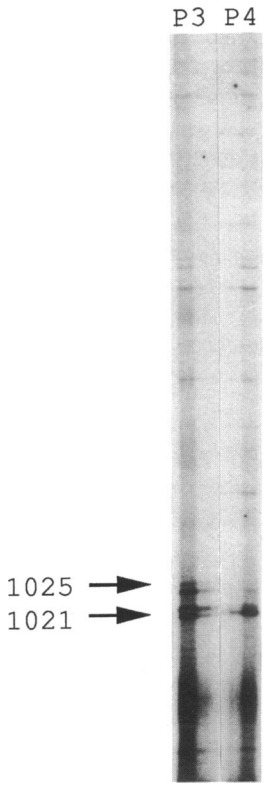

Figure 4 Primer extension analysis of 5′ end of LPH mRNA. An end-labeled, minus-strand primer complementary to nucleotides 87–68 of the LPH cDNA sequence was hybridized to intestinal mucosa RNA from subjects P3 and P4 and extended with reverse transcriptase. Aliquots of the products corresponding to 7 or 2 μg total RNA (outer and middle lanes, respectively) were analyzed by autoradiography after electrophoresis through a denaturing 6% polyacrylamide gel. The lengths of the products were determined by comparison with a dideoxy sequencing reaction carried out on a chromosomal LPH DNA template with the same primer (not shown). The 3′ end points of the products are given as positions in the sequence shown in fig. 3*A*.

template DNA, and the opposing PCR primer then used as sequencing primer. In parallel, the sequence for P2 was redetermined with this method to provide a side-by-side comparison.

Contrary to our expectation, the predicted amino acid sequence of H1 was essentially identical to that of P2, the only ambiguity arising from the unknown phase of two heterozygous positions (table 3).

cDNA Structure in a Hypolactasic Subject

We then asked whether alternative splicing and/or RNA editing might lead to different LPH sequences. Intestinal RNAs from subjects P3 and H2 were compared by an S_1-mapping procedure (Weaver and Weissmann 1979), using a set of overlapping end-labeled probes (see scheme in fig. 5*A*) derived from the chromosomal DNA of P2 and the cDNA of P1. The major protected fragments found were as expected on the basis of P1's cDNA (see scheme in fig. 5*A*), and there were no differences in fragment size between the tolerant and intolerant subject (fig. 5*B;* the quantitative differences are addressed below). These results demonstrate that no sequences present in the subjects with persistence of lactase are missing in the hypolactasic subject H2.

As the S_1-mapping method employed cannot reliably detect "extra" sequences in RNA (at the temperature we used, S_1 nuclease would not be expected to cut the probe DNA across from an unhybridized loop in the RNA; see Favaloro et al. 1980), we also compared P3 and H2 by analyzing overlapping segments of their respective cDNAs amplified by PCR (scheme in fig. 5*A*). No products longer than those expected on the basis of the cDNA sequence from P1 (Mantei et al. 1988) were seen (fig. 5*C*). With one pair of primers an exceptional amount of a shorter fragment was observed (fig. 5*C*, lanes h); this product probably arose because the plus-strand primer could also hybridize to a repeat (see fig. 6) within the LPH cDNA downstream of that intended. The S_1-mapping and PCR data together strongly suggest that LPH mRNA is spliced identically in P3 and H2. Subsequent sequence analysis (see below) also demonstrated the similarity of these LPH RNAs.

The amount of LPH RNA in H2 is typical of that given by Italian hypolactasic subjects studied in the earlier work (Sebastio et al. 1989), whereas P3 has substantially more LPH RNA than any subject we have previously studied (table 1). P3 was compared by S_1 mapping and PCR analysis with P4, a more typical subject with persistence of lactase, and identical protected and amplified fragments, respectively, were seen (data not shown).

Finally, the PCR products from the cDNA of H2 were directly sequenced as described for the chromosomal gene. P1, the subject whose LPH cDNA was originally characterized (Mantei et al. 1988), has rather low LPH activity (the ratio of sucrase to lactase activities is appropriate to persistence of lactase, but the activity of lactase is in the low range of values found for such subjects (see table 1; cf. Welsh et al. [1978] and Flatz [1989]). To provide a better comparison with H2, we therefore also sequenced the PCR products of two individuals with higher levels of lactase, P3 and P4 (see table 1). The sequences were es-

Table 3

Differences in LPH Sequences among Various Individuals with Adult-Type Hypolactasia and Persistence of Lactase

	P1		P2		P3		P4		H1		H2		
Nucleotide Position	Nucleotide Sequence	Predicted Amino Acid Sequence	Nucleotide Sequence	Predicted Amino Acid Sequence	Nucleotide Sequence	Predicted Amino Acid Sequence	Nucleotide Sequence	Predicted Amino Acid Sequence	Nucleotide Sequence	Predicted Amino Acid Sequence	Nucleotide Sequence	Predicted Amino Acid Sequence	Amino Acid Position
593.....	ACC	T	ACC/T	T	ACT	T	ACT	T	ACC/T	T	ACC	T	194
666.....	GTT	V	G/ATT	V/I	GTT	V	GTT	V	G/ATT	V/I	ATT	I	219
1124.....	AGA	R	AGG	R	AGG	R	AGG	R	AGG	R	AGG	R	371
1430.....	GGC	G	GGA/C	G	GGA	G	GGA	G	GGA/C	G	GGC	G	473
2468.....	CAC	H	CAC/T	H	CAC	H	CAC	H	CAC	H	CAC	H	819
3297.....	ACC	T	GCC	A	GCC	A	GCC	A	GCC	A	GCC	A	1096
4340.....	GGT	G	GGC	G	GGC	G	GGC	G	GGC	G	GGC	G	1443
4508.....	ACG	T	ACG	T	ACG	T	ACG/T	T	ACG	T	ACG	T	1499
4617.....	TTG	L	C/TTG	L	TTG	L	TTG	L	C/TTG	L	CTG	L	1536
4927.....	AAT	N	AA/GT	N/S	AGT	S	AGT	S	AA/GT	N/S	AAT	N	1639
5579.....	GCT	A	GCT/C	A	GCC	A	GCC	A	GCT/C	A	GCT	A	1856
5845.....	C		C/G		C		C		C/G		G		
6236/37.....	TG		TG/ΔΔ		TG		TG		TG/ΔΔ		ΔΔ		
6261.....	A		C		A		. . .		C		. . .		

NOTE.—Predicted amino acid sequences are in single-letter code. For subject P1, one allele is presented; in other cases both alleles were sequenced. Codons which differ are shown, with heterozygous positions indicated by underlining and slashes. Positions beyond 5792 are in the 3′-untranslated region. Δ = deletion; . . . = not determined. Nucleotide and amino acid positions are numbered as in the LPH cDNA (Mantei et al. 1988).

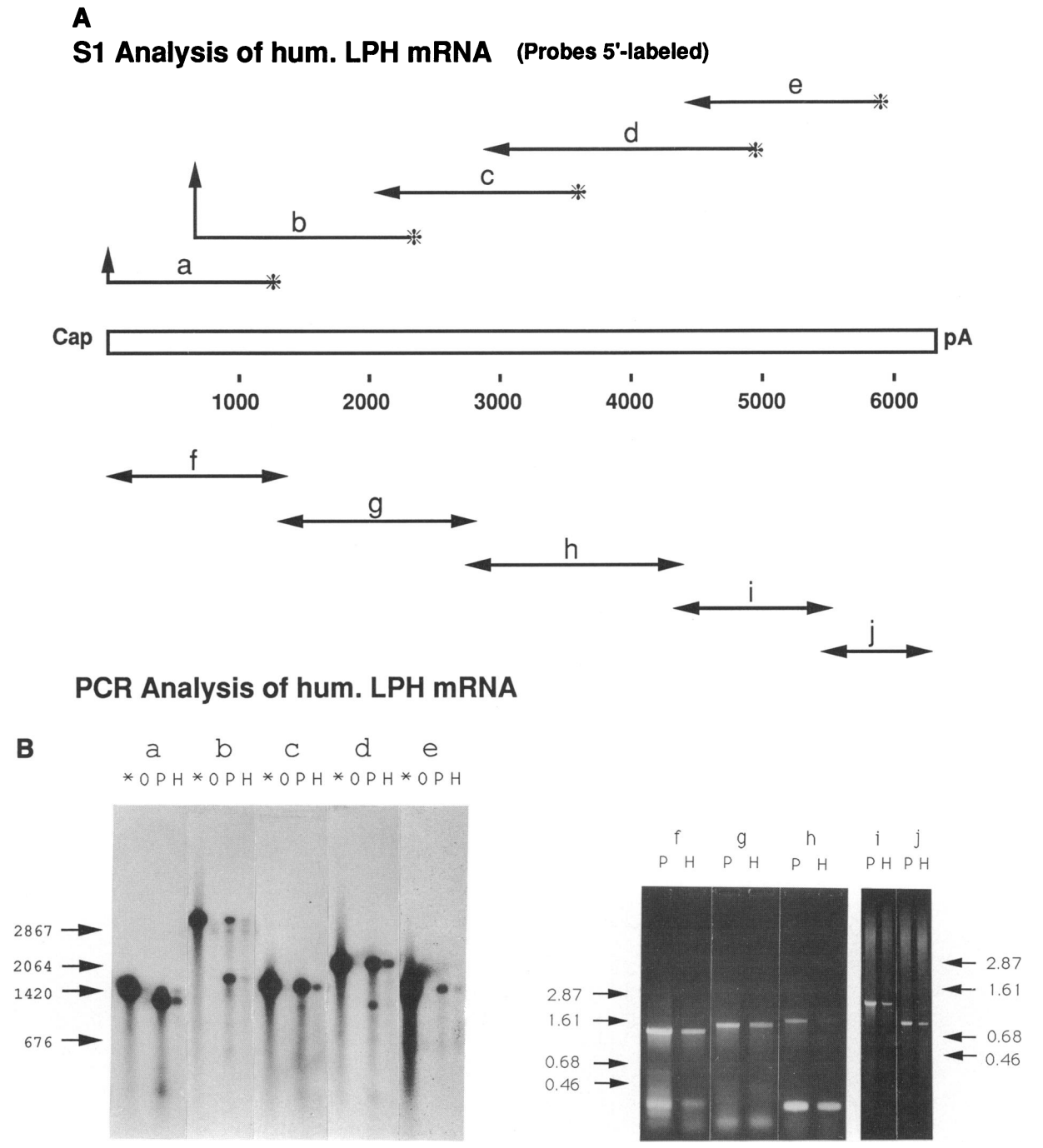

Figure 5 S_1-mapping and PCR analysis of LPH RNA in subjects with adult-type hypolactasia and persistence of lactase. *A*, Scheme of analysis. The LPH mRNA is represented by the open box. End-labeled probes a–e for S_1 mapping are as follows (asterisks indicate the labeled sites, and the numbers in parentheses the locations of the sites within the LPH cDNA sequence); a, *Bam*HI*-(1248)–*Pvu*II from pHlac61 (Mantei et al. 1988); b, *Acc*I*(2328)–*Hin*dIII from pHlacexp (W. Boll, unpublished data); c, *Hin*dIII*(3576)–*Ava*I from pHlac61; d, *Bam*HI*(4949)–*Pvu*II from pHlac61; e, oligonucleotide no. 62 (table 2), end-labeled and then extended in single-stranded PCR on a pHlac61 template cleaved with *Cla*I. The vertical portions of the arrows indicate probe sequences not homologous to LPH cDNA. For PCR analysis, first-strand cDNA prepared from intestinal RNA was amplified with the following pairs of primers: f, 52 and 14; g, 15 and 22; h, 23 and 58; i, 59 and 63; j, 62 and 64, where the numbers refer to table 3. *B*, S_1 mapping. The various probes (a–e; see scheme in part *A*), end labeled to about 1–6 × 10^6 cpm/pmol, were hybridized with 1 μg total intestinal mucosa RNA or yeast RNA in 80% formamide buffer. Following digestion with S_1 nuclease, the products were separated by electrophoresis in a 1.3% alkaline agarose gel and autoradiographed. Lanes *, probe alone (no S_1); lanes 0, yeast RNA; lanes P, RNA from P3; lanes H, RNA from H2. *C*, PCR amplification. First-strand cDNA prepared from mucosa RNA from subjects P3 (lanes P) and H2 (lanes H) was amplified by PCR using the primer pairs described above (f–j; see scheme in part *A*), and the products electrophoresed in a 1.2% agarose gel. (cDNA synthesis primed with random hexanucleotides for lanes f–h and with oligo$(dT)_{12-18}$ for lanes i and j.)

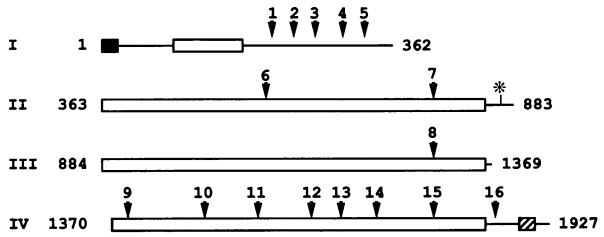

Figure 6 Distribution of introns within repeats of LPH gene. The four homologous regions within the LPH cDNA (cf. fig. 4 of Mantei et al. 1988) are indicated by open boxes, and regions with no recognizable homology by lines. The black box represents the region coding for the signal sequence, the striped box that coding for the hydrophobic membrane anchor, and the first amino acid of mature LPH (amino acid 869 of pre-pro-LPH) is indicated by an asterisk. The positions of the introns, 1–16, are indicated by vertical arrows.

sentially identical to those determined previously for P1, P2, and H1. The LPH cDNA of H2 was found to be completely homozygous, and no new alleles beyond those seen in variable positions in other subjects previously characterized (table 3) were found. We conclude that the subjects examined code for an essentially identical LPH enzyme.

Discussion

Structure of the Human Chromosomal LPH Gene

Based on S_1-mapping data using end-labeled probes spanning the cap site (Sebastio et al. 1989; and W. Boll, unpublished data) and on a primer extension experiment (fig. 4), we have assigned the cap site(s) to the region 1022–1026 in the sequence shown in figure 3*A*. In addition to a TATA element 23–27 nucleotides upstream of this position, a search for other known eukaryotic promoter elements (for reviews see Johnson and McKnight 1989; Mitchell and Tjian 1989) revealed good matches to consensus binding sites for Sp1 (814–819), SRF (773–781), AP-2 (615–621), CTF/NF-1 (483–487, and also within the coding region at positions 116–120 of the cDNA sequence [Mantei et al. 1988]), CREB (386–392), and Oct1/Oct2 (nucleotides 42–48) (fig. 3*A*). However, functional tests of these elements have not yet been carried out with the LPH gene. Two *Alu* repeats (Deiniger et al. 1981) are found in this region, at nucleotides 177–481 and 571–871, and the Sp1, SRF, Ap-2, and CREB elements are within the repeats. A consensus binding site for glucocorticoid receptor was *not* found. Although glucocorticoid hormones have been found to stimulate LPH production in organ culture of human fetal intestine (Simon-Assmann et al. 1984; Arsenault and Ménard 1985), no significant increase in LPH RNA levels was detected upon hydrocortisone treatment of such organ cultures (M. Villa, D. Ménard, N. Mantei, and G. Semenza, unpublished data), suggesting that glucocorticoids do not directly stimulate transcription of the LPH gene.

As mentioned in the introduction, the LPH cDNA is fourfold internally repeated, and probably arose through two cycles of gene duplication (Mantei et al. 1988). One would expect "primordial" introns, present before any duplication, to be present at homologous positions within the repeats. As shown in figure 6, only one set of introns (7, 8, and 15) fulfills this prediction. This would suggest that many introns have been gained or lost during the evolution of the LPH gene.

Comparison of LPH Gene and mRNA Structure in Adults with Hypolactasia and Persistence of Lactase

Can adult-type hypolactasia and persistence of lactase be correlated with differences in LPH sequences? As shown in table 3, sequence differences were found in 14 positions, 11 of these being in the coding region and only three leading to changes in amino acid sequence. The two alleles at amino acid position 1096, coding for threonine or alanine, do not correlate with hypolactasia, as the two hypolactasic subjects are homozygous for an allele also found in homozygous form in subjects P2, P3, and P4. In the case of the valine/isoleucine allelism at position 219, as well as the asparagine/serine allelism at position 1639, subjects P2 and H1 carry both alleles at each position. As persistence of lactase is dominant, if either allele at either single position were important for distinguishing hypolactasia from persistence of lactase, subject H1 would necessarily have persistence of lactase, which is not the case. Thus, these allelic differences can be correlated with persistence of lactase only if a particular allele at *both* positions is required. Considering that the changes in amino acids at each position are conservative, the possibility that the linkage phase is important seems remote.

The sequences of LPH cDNAs from subjects H2, P3, and P4 were determined by sequencing overlapping PCR products derived from cDNA. Comparison of these sequences and that of LPH cDNA from subject P1 (Mantei et al. 1988) showed that also at the mRNA level there were no significant differences in LPH sequences, such as might have arisen through alternative splicing of RNA editing. Further, LPH RNA from subjects P3 and P4 was compared with that from subject H2 by S_1 mapping and by PCR analysis of the respec-

tive cDNAs; here again we found no evidence for alternative splicing.

Finally, Southern blot analysis, and the fact that all our genomic LPH clones fall into one linkage group, show that there is only one LPH gene which is closely related to the cDNA of subject P1. As the residual LPH produced by hypolactasic humans has been reported to be similar or identical to that of subjects with persistence of lactase (Lorenz-Meyer et al. 1972; Crane et al. 1976; Skovbjerg et al. 1980; Potter et al. 1985), more distantly related genes are unlikely to play a role in hypolactasia versus persistence of lactase. All the above evidence leads us to conclude that subjects with persistence of lactase and hypolactasic subjects can code for identical pre-pro-LPH.

Decline of LPH Activity in Adult-Type Hypolactasia

The decline in LPH activity occurring in late childhood or early youth in many, but not all, human populations leads to adult-type hypolactasia or lactose intolerance. No other enzymes of the brush border membrane are known to be developmentally regulated in this fashion.

We have previously compared LPH RNA levels among subjects of Italian descent (Sebastio et al. 1989) and found a poor correlation between LPH RNA and enzyme levels. Hypolactasic subjects had, on the average, 50% as much LPH RNA as subjects with persistence of lactase, and at least one had more than the average of the subjects with high lactase. In the present work we noted that subject P3 has more than 50 times more LPH RNA than P1, yet has only twice as much LPH enzymatic activity (table 1; the RNA from subject P1 was not merely degraded, as judged by agarose gel electrophoresis and the fact that the original 6 kb cDNA clones were readily prepared from this material). On the other hand, subject H2 has about 70% as much LPH mRNA as P4, but only 11% as much lactase activity (table 1). Although this remarkably poor correlation between mRNA and enzyme level may in part reflect differences in the samples (e.g., location along the length of the intestine), at least for the subjects studied by us (Sebastio et al. 1989; the present work) the weight of the evidence favors post-transcriptional regulation. We note further that, as there is only one LPH gene (see above), transcription from a pseudogene cannot be invoked to explain the high levels of LPH mRNA seen in some lactose-intolerant subjects.

This regulation could be at the translational level or act at some posttranslational step. Pulse labeling of LPH in organ cultures of human intestine (Sterchi et al. 1990; Witte et al. 1990) showed that in most (but not all) cases the amount of LPH precursor was reduced in hypolactasia, even with labeling times as short as 30 min. Several possibilities may be envisaged to account for these results. (1) The levels of LPH RNA may be low in subjects with low synthesis. Analysis of mRNA levels and biosynthesis of LPH in the same subjects should answer this question. (2) The LPH RNA of the hypolactasic subjects may be poorly translated. (3) The newly synthesized pro-LPH may be rapidly degraded (within 30 min), perhaps within the endoplasmic reticulum or the Golgi. As the time required for synthesis of mature LPH and its transport to the brush border membrane is 3 h or more (Naim et al. 1987), extracellular degradation cannot explain the observations. We have seen above that the pre-pro-LPH of subjects with hypolactasia and with persistence of lactase is identical. Thus we can exclude models which explain the decline in terms of subtle differences in primary sequence leading, for example, to unstable LPH. The further observation of slow processing of LPH precursor in hypolactasia (Sterchi et al. 1990; Witte et al. 1990), including a case in which slow processing was the only detectable variation (Witte et al. 1990), suggests that the decline of LPH may have multiple causes, perhaps varying among populations. Our own results in combination with these and other studies raise the interesting possibility that at least some cases of adult-type hypolactasia may reflect an unusual mechanism involving (1) a developmentally regulated change in protein processing or targeting which (2) does not occur in subjects with persistence of lactase, (3) affects specifically LPH and not the other brush border glycosidases, and (4) does not depend for its specificity on an LPH sequence different from that found in persistence of lactase.

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appear in the EMBL/GenBank/DDBJ nucleotide sequence data bases under accession numbers M61834–M61850.

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