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Mapping the Human Amylase Gene Cluster on the Proximal Short Arm of Chromosome 1 Using a Highly Informative $(CA)_n$ Repeat

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The human amylase gene cluster includes a $(CA)_n$ repeat sequence immediately upstream of the γ-actin pseudogene associated with the AMY2B gene. Analysis of this $(CA)_n$ repeat by PCR amplification of genomic DNA from the 40 families of the Centre d'Etude du Polymorphisme Humain (CEPH) reference panel revealed extensive polymorphism. A total of six alleles with $(CA)_n$ lengths of 16–21 repeats were found. The average heterozygosity for this polymorphism was 0.70. Multipoint linkage analysis showed that the amylase gene cluster is located distal to the nerve growth factor β-subunit gene (NGFB) and is within 1 cM of the anonymous locus D1S10. The amylase $(CA)_n$ repeat provides a convenient marker for both the physical and the genetic maps of human chromosome 1p. © 1990 Academic Press, Inc.

INTRODUCTION

The human amylase gene cluster spans 240 kb and includes two pancreatic amylase genes (AMY2A and AMY2B), three salivary amylase genes (AMY1A, AMY1B, and AMY1C), and a truncated pseudogene (AMYP1) (Gumucio *et al.*, 1988; Samuelson *et al.*, 1988; Groot *et al.*, 1989). The amylase genes have been mapped to chromosome 1p21 (Munke *et al.*, 1984; Tricoli and Shows, 1984; Zabel *et al.*, 1983). Extensive analysis of this gene cluster revealed only two relatively uninformative RFLPs (Gumucio *et al.*, 1985; Ishizaki *et al.*, 1985). In a search for a more informative polymorphic marker in the amylase gene cluster, we have analyzed the $(dC\text{–}dA)_n \cdot (dG\text{–}dT)_n$ or $(CA)_n$ repeat sequence located 48 nucleotides upstream of the γ-actin pseudogene ACPGP3 and 2.3 kb upstream of AMY2B (Samuelson *et al.*, 1990). Approximately 10^5 copies of the tandemly repeated DNA $(CA)_n$ sequences are interspersed throughout the human genome (Miesfield *et al.*, 1981; Hamada and Kakunaga, 1982; Hamada *et al.*, 1982). These $(CA)_n$ repeats have recently been shown to be highly polymorphic (Weber and May, 1989; Litt and Luty, 1989) and provide a useful new source of polymorphisms for genetic analysis.

MATERIALS AND METHODS

Families

A total of 526 genomic DNA samples from the 40 families of the CEPH reference pedigrees (Dausset, 1986) were used in the analysis of the $(CA)_n$ repeat sequence in the human amylase gene cluster.

PCR Analysis

The $(CA)_n$ repeat which is located 5′ to the AMY2B gene (Fig. 1) occurs in an AT-rich region. The two primers were selected to maximize the GC content and, where possible, to have at least two G's or C's at the 3′ end of the oligonucleotide. The coding strand primer is a 25-mer (5′-TATTTACTGTCCTTATTTATGT-GGG-3′). The noncoding strand primer is a 20-mer (5′-AAACCTCTGGCAGTGTACAC-3′). Primers were synthesized by the University of Michigan Center for Molecular Genetics.

The coding strand primer was end-labeled for 30 min at 37°C in a 25-μl reaction containing 20 μl [γ-^{32}P]ATP (6000 Ci/mmol), 1 μl primer (20 μ*M*), 2.5 μl 10× kinase buffer (1× = 50 m*M* Tris (pH 7.6), 10 m*M* $MgCl_2$, 5 m*M* DTT, 100 μ*M* spermidine, 100 μ*M* EDTA), and 1 μl T4 polynucleotide kinase (50 U/μl). The T4 polynucleotide kinase was inactivated by incubation at 65°C for 10 min. The entire 25-μl kinase reaction, without separating the unincorporated nucleotides, was added to sufficient buffer mix for 25 to 100 PCR reactions.

PCR was performed under standard conditions (Saiki *et al.*, 1985, 1988) in 25-μl reactions containing 1 μl genomic DNA template (200 ng), 1.25 μl each oligonucleotide primer (20 μ*M*), 4 μl dNTP mix (1.25 m*M* each dATP, dCTP, dGTP, dTTP), 2.5 μl 10× PCR

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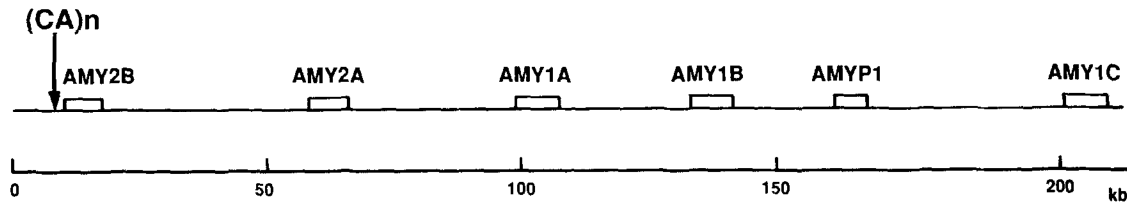

FIG. 1. The $(CA)_n$ repeat block is located 2.3 kb upstream of the human amylase gene cluster. This cluster includes two pancreatic amylase genes (AMY2A and AMY2B), three salivary genes (AMY1A, AMY1B, and AMY1C), and one truncated gene (AMYP1) (9, 11). Not shown but also present within the 240-kb region are multiple copies of a γ-actin processed pseudogene and an endogenous retrovirus (31).

buffer (1× = 10 m*M* Tris (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin), 0.125 μl AmpliTaq DNA polymerase (5 u/μl), and 0.25–1.00 μl primer end-labeling reaction mix. For amplification, each sample was denatured (3 min at 94°C) and subjected to 30 amplification cycles (each cycle consisting of 2 min annealing at 55°C, 3 min extension at 72°C, and 1 min denaturing at 94°C) in a Perkin–Elmer–Cetus thermal cycler. The amplified DNA was run on 8% acrylamide sequencing gels. The gels were not dried, but only covered with a thin plastic film and exposed at −70°C to Kodak XAR-5 film for 2–24 h.

The sizes of the $(CA)_n$ repeats in the genomic DNA samples were determined by comparison with that of a cloned $(CA)_{21}$ repeat from this region (Samuelson *et al.,* 1990).

Linkage Analysis

Genotypes of the AMY2B $(CA)_n$ repeat were entered into a database containing genotypes from a large number of chromosome 1 polymorphisms typed on the CEPH pedigrees (Dracopoli *et al.,* 1988b; O'Connell *et al.,* 1989). The α-satellite centromeric repeat polymorphism (D1Z5) (Waye *et al.,* 1987) genotypes in the CEPH database (M. Mahtani, R. Lafreniere, and H. Willard, unpublished data) were used with permission of Dr. H. Willard. Initial analyses and data checking were carried out using the CEPH software package. The AMY2B $(CA)_n$ genotypes were converted into MAPMAKER (Lander *et al.,* 1987) format using the LNKTOMAP program provided by Dr. K. Buetow. All two-point and multipoint linkage analyses were performed using MAPMAKER. All analyses were performed on a SUN 386i workstation.

RESULTS

Polymorphism at the AMY2B $(CA)_n$ Repeat

The $(CA)_n$ repeat is located 2.3 kb upstream of the AMY2B locus at the 5′ end of the amylase gene cluster (Fig. 1). To detect polymorphism, we used PCR primers corresponding to sequences on either side of the $(CA)_n$ repeat. The resulting pattern of amplified DNA consisted of multiple bands of varying intensity (Fig. 2). These multiple bands have been detected in other $(CA)_n$ repeats and are presumed to be the result of the polymerase "stuttering" as it synthesizes the complementary strand of the $(CA)_n$ repeat during the PCR amplification (Weber and May, 1989). Homozygotes contain a single intense band with approximately four additional bands of diminishing intensity decreasing in size in single nucleotide steps (see lane 2 in Fig. 2). The largest band is always the most intense band in homozygotes. Heterozygotes contain two series of bands. These are easy to distinguish when the alleles differ in size by at least 4 nucleotides. However, the series of bands are superimposed in heterozygotes where the allele sizes differ by only 2 nucleotides. In these cases, it is possible to identify heterozygotes by the relative band intensities resulting from the superimposition of bands from the smaller allele on the trailing bands of the larger allele (see lane 1 in Fig. 2).

Six alleles were identified in the 526 samples of the CEPH pedigree (Table 1). The sizes of these alleles all differed by 2 nucleotides, suggesting that each allele

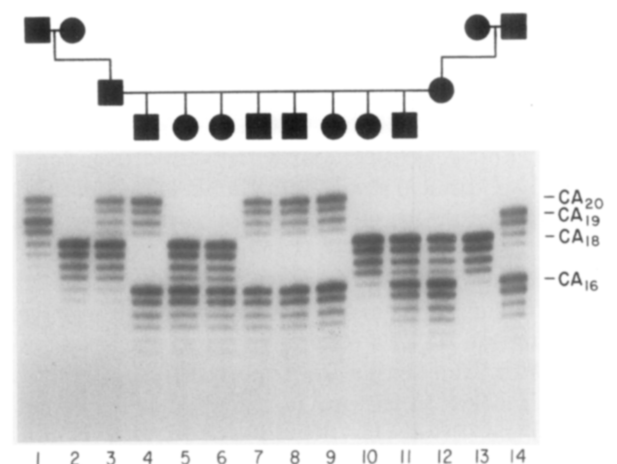

FIG. 2. $(CA)_n$ genotypes in CEPH family 1323. The pedigree is superimposed above the gel and the number of dinucleotide repeats in each allele is indicated on the right. The genotypes (defined by the number of CA dinucleotides) for each individual in the pedigree are as follows: Lane **1,** 19/20; **2,** 18/18; **3,** 18/20; **4,** 16/20; **5,** 16/18; **6,** 16/18; **7,** 16/20; **8,** 16/20; **9,** 16/20; **10,** 18/18; **11,** 16/18; **12,** 16/18; **13,** 18/18; **14,** 16/19.

TABLE 1

Allele Frequencies of the $(CA)_n$ Repeat at the AMY2B Locus

$(CA)_n$	Frequency
$(CA)_{16}$	0.02 ± 0.01
$(CA)_{17}$	0.03 ± 0.01
$(CA)_{18}$	0.46 ± 0.04
$(CA)_{19}$	0.28 ± 0.04
$(CA)_{20}$	0.16 ± 0.03
$(CA)_{21}$	0.06 ± 0.02

Note. The allele frequencies were determined by typing all 80 parents of the CEPH reference panel.

was generated by the gain or loss of a single CA dinucleotide. The amplified product between the two primers varied between 76 and 86 nucleotides. Complete concordance with Mendelian segregation was observed in all 40 of the CEPH families, and at least one parent was heterozygous in 38 families. The alleles ranged in size from $(CA)_{16}$ to $(CA)_{21}$. The allele frequencies are not evenly distributed, and the three intermediate repeats, sizes $(CA)_{18-20}$, account for approximately 90% of the alleles at this locus. The observed heterozygosity at this locus was 0.70 and the polymorphic information content (Botstein *et al.*, 1980) was 0.63. The allele frequencies did not differ from Hardy–Weinberg equilibrium ($\chi^2 = 8.31$, $P > 0.99$). The genotypes of the AMY2B $(CA)_n$ repeat in the 40 CEPH reference families have been deposited in the CEPH database.

Linkage Analysis

Preliminary two-point linkage analysis showed strong linkage for the AMY2B $(CA)_n$ repeat with a large number of markers from proximal 1p (Table 2). The closest marker appeared to be locus D1S10, which showed only 1% recombination with a Lod score of 32.8. In the first stage of multipoint analysis, the AMY2B $(CA)_n$ polymorphism was tested against a fixed map of chromosome 1 that had been developed using the CEPH families (Dracopoli *et al.*, 1988b; O'Connell *et al.*, 1989). This analysis showed that AMY2B was located very close to D1S10 in the interval between D1S12 and D1S14. The relative orientation of AMY2B and D1S10 could not be determined.

The multipoint map of this region was recalculated after the addition of the AMY2B $(CA)_n$ polymorphism because the addition of a highly informative locus to a fixed map may affect the distances and relative order of tightly linked loci. The 120 possible orders of AMY2B, D1S9, D1S10, D1S12, and D1S14 were compared while four distal loci (PGM1, D1S22, D1S16, D1S20) and four proximal loci (D1S11, TSHB, NGFB, NRAS) were held constant (Table 3). This analysis showed that the addition of the AMY2B $(CA)_n$ repeat affected the relative orientation of the two nearest proximal loci (D1S10 and D1S14). Previous analysis of this region tentatively placed D1S14 less than 2 cM distal to D1S10 with support levels less than 100:1 (Dracopoli *et al.*, 1988b). The inclusion of AMY2B in this map reverses the orientation of D1S14 and D1S10 and provides strong support (>5000:1) that the tightly linked cluster of D1S10–AMY2B is located distal to D1S14 (Table 3). The relative orientation of AMY2B and D1S10 cannot be resolved. Examination of the CEPH families shows only two recombinants between AMY2B and D1S10 in individuals 1332-03 and 1408-03. The multipoint map of proximal chromosome 1p is shown in Fig. 3.

TABLE 2

Summary of Two-Point Lod Scores for the AMY2B $(CA)_n$ Repeat and 14 Polymorphic Loci from Proximal Chromosome 1p

Locus	θ	θ_m	θ_f	LOD
D1S9	0.09	0.03	0.14	27.08
D1S10	0.01	0.02	0.01	32.82
D1S11	0.13	0.04	0.17	16.09
D1S12	0.06	0.02	0.10	20.49
D1S13	0.22	0.25	0.20	7.34
D1S14	0.08	0.12	0.04	26.01
D1S16	0.21	0.25	0.20	6.82
D1S20	0.11	0.13	0.10	6.14
D1S22	0.38	0.36	0.40	0.54
NGFB	0.14	0.05	0.22	24.69
NRAS	0.11	0.05	0.25	19.12
PGM1	0.36	0.30	0.50	2.28
TSHB	0.17	0.12	0.21	20.98

DISCUSSION

A human amylase serum polymorphism was originally mapped to chromosome 1 by linkage to the un-

TABLE 3

Multipoint Analysis of the Four Most Likely Orders of AMY2B, D1S9, D1S10, D1S12, and D1S14

Possible orders	Relative likelihood
D1S12–AMY2B–D1S10–D1S14–D1S9	—
D1S12–D1S10–AMY2B–D1S14–D1S9	1:1.4
D1S12–AMY2B–D1S14–D1S10–D1S9	1:7,390.1
D1S10–AMY2B–D1S12–D1S14–D1S9	1:18,505.9

Note. These orders were determined by calculating the 120 possible orders of the five loci (AMY2B, D1S9, D1S10, D1S12, and D1S14) while the locations of the four distal (PGM1–D1S22–D1S16–D1S20) and the four proximal markers (D1S11–TSHB–NGFB–NRAS) were held constant.

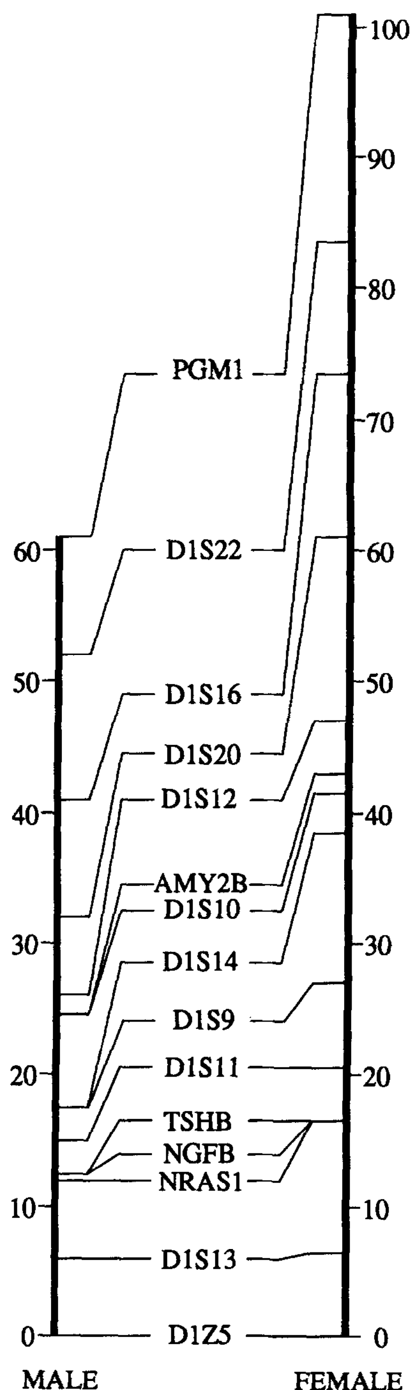

FIG. 3. Multipoint linkage map of proximal chromosome 1p from PGM1 to D1Z5. The recombination rates in each interval were permitted to differ between sexes, and the map distances are calculated using the Kosambi mapping function. The most favored map order is presented.

coiler region (Kamaryt *et al.*, 1971) and to the Duffy blood group locus (Hill *et al.*, 1972; Merritt *et al.*, 1972, 1973). Assignment to the pericentromeric region of chromosome 1 was based on linkage analysis of a variety of blood group antigens, red cell enzymes, and serum proteins (Robson and King, 1984; Sherman *et al.*, 1984; Wedd, 1984). The position of the amylase cluster was refined to 1p21–p22 by Southern blot analysis of somatic cell hybrids (Tricoli and Shows, 1984; Munke *et al.*, 1984) and localized to 1p21 by *in situ* hybridization (Zabel *et al.*, 1983; Munke *et al.*, 1984).

The map of the proximal region of chromosome 1 has been confused for a number of years by conflicting physical assignments of the genes for the β-subunit of nerve growth factor (NGFB), the β-subunit of thyroid stimulating hormone (TSHB), and the neuroblastoma RAS viral oncogene homolog (NRAS). NGFB has been assigned to 1p22.1 (Zabel *et al.*, 1985; Francke *et al.*, 1983; Munke *et al.*, 1984) and to 1p13 (Garson *et al.*, 1987). TSHB has been assigned to 1p22 (Dracopoli *et al.*, 1986) and has been shown to be on the same 310-kb *Sfi* fragment as NGFB (Dracopoli *et al.*, 1988a). NRAS has been assigned to 1p13 (Davis *et al.*, 1984; Garson *et al.*, 1987) and to 1p22 (Munke *et al.*, 1984).

It is evident from linkage analysis and pulsed-field gel electrophoresis that TSHB, NGFB, and NRAS form a very tightly linked gene cluster and that they must all be assigned to the same chromosomal band. Their location proximal to the AMY2B gene in 1p21, and close linkage to the α-satellite centromeric repeat (D1Z5) (Fig. 3) provide strong evidence that the correct assignment for these three loci is 1p13 and not 1p22. Therefore the independent assignments of TSHB (Dracopoli *et al.*, 1986) and NGFB and NRAS (Munke *et al.*, 1984) to 1p22 are most likely incorrect. TSHB was assigned to chromosome 1p22 by analysis of somatic cell hybrids with breakpoints on proximal 1p. The proximal breakpoint was defined by cell line A9/1492. It seems likely that the cytogenetic analysis of this hybrid was incorrect and that A9/1492 contains the region 1p13–pter and does not break in 1p21 as previously thought (Rettig *et al.*, 1984, 1986; Dracopoli *et al.*, 1986, 1988a,b). Consequently, chromosomal assignments based on this breakpoint should be redefined and the smallest region of overlap (SRO) for the loci MSK1 (Rettig *et al.*, 1984, 1986), D1S9, D1S10, D1S11, D1S12, D1S13, D1S14 (Dracopoli *et al.*, 1988b), and TSHB (Dracopoli *et al.*, 1986) should be extended to cover the region 1p22–p13. Examination of the linkage map of chromosome 1 further demonstrates that TSHB and D1S13 should be assigned to 1p13 and that D1S9, D1S10, D1S11, D1S12, and D1S14 should be assigned to 1p13–p22.

The amylase gene cluster has been the site of multiple insertions, deletions, and duplications during primate evolution (Samuelson *et al.*, 1990). The paucity of RFLPs detected with amylase probes may reflect the recent origin of multiple gene copies from a single ancestral gene. The high PIC of the amylase-associated $(CA)_n$ repeat demonstrates the utility of this type of variation for human linkage studies, as was proposed in two recent reports (Weber and May, 1989; Litt and

Luty, 1989). The detection of (CA)$_n$ polymorphism by PCR is faster, less laborious, and less expensive, requires less genomic DNA, and is likely to be more informative than the standard analysis of RFLPs by Southern blotting. These practical advantages justify an effort to identify additional polymorphisms of this type for use in human linkage analyses.

It has recently been suggested that the physical map of the human genome could be anchored by a reference set of PCR primers that detect unique sites in the human genome (Olson *et al.*, 1989). The primers described here can serve this function both as an anchor point for the physical map of chromosome 1p and as a connection to the linkage map of 1p through the (CA)$_n$ polymorphism.

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