Mutations in the RNA Component of RNase MRP Cause a Pleiotropic Human Disease, Cartilage-Hair Hypoplasia

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Summary

The recessively inherited developmental disorder, cartilage-hair hypoplasia (CHH) is highly pleiotropic with manifestations including short stature, defective cellular immunity, and predisposition to several cancers. The endoribonuclease RNase MRP consists of an RNA molecule bound to several proteins. It has at least two functions, namely, cleavage of RNA in mitochondrial DNA synthesis and nucleolar cleaving of pre-rRNA. We describe numerous mutations in the untranslated RMRP gene that cosegregate with the CHH phenotype. Insertion mutations immediately upstream of the coding sequence silence transcription while mutations in the transcribed region do not. The association of protein subunits with RNA appears unaltered. We conclude that mutations in RMRP cause CHH by disrupting a function of RNAse MRP RNA that affects multiple organ systems.

Introduction

Pleiotropy, that is, multiple phenotypic manifestations of a single genetic defect, is a hallmark of McKusick-type metaphyseal chondrodysplasia or cartilage-hair hypoplasia (CHH) [MIM #250250] (McKusick et al., 1965). The most prominent characteristic of this recessively inherited disorder is disproportionate short stature with adult heights ranging from 104 to 149 cm. Other common features include hypoplastic hair, ligamentous laxity, defective immunity, hypoplastic anemia, and neuronal dysplasia of the intestine. Manifestations occurring in a smaller proportion of affected individuals include congenital megacolon (Hirschsprung’s disease), and predisposition to lymphoma and other cancers. The variation in clinical severity is remarkable both between and within sibships (McKusick et al., 1965; Mäkitie, 1992; Mäkitie and Kaitila, 1993). CHH was first described in the Old Order Amish where it is a common cause of short-limbed short stature (McKusick et al., 1965; McKusick, 2000). The incidence among the Amish was calculated as 1 in 1340 implicating a carrier frequency as high as 1:19 (Sulisalo et al., 1994a). Another founder population, the Finns, displays a similar gene enrichment with a calculated carrier frequency of 1:76 (Mäkitie, 1992). Cases have been described in numerous other populations; however, there is no precise measure of its worldwide incidence (Mäkitie, 1992). A locus responsible for CHH was mapped to a region on the short arm of chromosome 9 eight years ago (Sulisalo et al., 1993), and subsequently shown to account for most or all CHH (locus homogeneity) (Sulisalo et al., 1994a, 1995). The critical region has been reduced to less than 1 Mb of DNA by linkage and linkage disequilibrium analysis (Sulisalo et al., 1994b) and physical mapping (Vakkilainen et al., 1999). However, in spite of intensive study of candidate genes in the critical region (Ben-Yosef and Francomano, 1999; unpublished data by the present authors) the gene has been elusive (McKusick, 2000).

Here we describe mutations in the RMRP gene causing CHH. This study describes disease-causing mutations in a nuclear gene encoding a structural RNA molecule. The untranslated RMRP gene is transcribed by RNA polymerase III, encodes the RNA component of a ribonucleoprotein endoribonuclease (Topper and Clayton, 1990), and is located in the CHH critical region (Hsieh et al., 1990 and results shown here). Normally the RNase MRP complex is involved in multiple cellular and mitochondrial functions. In the nucleolus this endonuclease partakes in the processing of pre-rRNA whereas in the mitochondrion it cleaves the RNA primers responsible for DNA replication (Chang and Clayton, 1989; Clayton, 1994; reviewed by van Eenennaam et al., 2000). As we did not find any disturbances in the association of specific proteins with RNase MRP RNA from CHH patients, the disease-causing functional impairment of the RMRP gene product remains to be characterized. The existence of one or more elusive additional substrates for this endonuclease has been postulated (Tollervey and Kiss, 1997; Cai et al., 1999). Our findings provide a first step toward an improved understanding of these processes leading to the pleiotropic phenotypic manifestations of CHH.
Table 1. The New Polymorphic Markers Used in Genetic Mapping. The Order Is from the Telomere to the Centromere

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Type of Polymorphism</th>
<th>PCR Primer Sequences (5’→3’</th>
<th>PCR Product Size (bp)</th>
<th>SSCP Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>16P1</td>
<td>Centromeric to AB002373 dup</td>
<td>TGCATGCA</td>
<td>AAGGATCCCTCGCAGCGCTTTC GCACCCGTGCTAATCTGTTTCT</td>
<td>182/190</td>
<td>6% Sequagel</td>
</tr>
<tr>
<td>AB12GS</td>
<td>AB002373</td>
<td>A/G</td>
<td>AGTGGGCCCCATAGGAAACAC CCCCTTTGAGTGGTTGGTCCATAC</td>
<td>118</td>
<td>0.7× MDE</td>
</tr>
<tr>
<td>Del13</td>
<td>CD72</td>
<td>13 nt deletion</td>
<td>GAGGCCATGGGGAGTCCACAG GTGGAAGACACTCTTGACCACTCCAGGCAG</td>
<td>103/90</td>
<td>2% SeaKem</td>
</tr>
<tr>
<td>SIT45S</td>
<td>SIT intron 3</td>
<td>T/C</td>
<td>CGGTCTGACGCTGCTTATTTC AATCGAGGTCCCTCCATACACGC</td>
<td>223</td>
<td>0.5× MDE</td>
</tr>
<tr>
<td>TP2A</td>
<td>TPM2 exon 2A</td>
<td>G/A</td>
<td>CGCTTCTCCCTCTGACATAG GACAGAGGGGACCTTGTCAG</td>
<td>270</td>
<td>0.7× MDE</td>
</tr>
<tr>
<td>TA13S</td>
<td>nt 3860 in TLN cDNA</td>
<td>C/T</td>
<td>CTGTTGACGCTGCTACCCTTG AGAGGCCTGCGCATTTCTTG CATCCTCTCCCTCGAAGAATGAC</td>
<td>143</td>
<td>1× MDE</td>
</tr>
<tr>
<td>D4S</td>
<td>KIAA0582</td>
<td>G/A</td>
<td>AAACCTCAGGGGCTGCATGCAC GACGACGCGACGTAACCCCTC</td>
<td>187</td>
<td>0.8× MDE</td>
</tr>
<tr>
<td>D6S</td>
<td>KIAA0582</td>
<td>G/A</td>
<td>AAATGAGATCCATACACCCCTC CGACGTCTCCCTCCCCACAT</td>
<td>137</td>
<td>0.7× MDE</td>
</tr>
<tr>
<td>CAI</td>
<td>Between 37D7T7 and Z38986 AC repeat</td>
<td>TTTCTTGCTGTGTTGGTGTTG</td>
<td>TTTCTTGCTGTGTTGGTGTTG</td>
<td>~140</td>
<td>6% Sequagel</td>
</tr>
<tr>
<td>XL6S</td>
<td>Telomeric to EST-C</td>
<td>G/A</td>
<td>AAAGGCGAGCGTCGCGGTTTTC AGACTGAGTCAGAGGGAGAAG CGCAAGGCTGTTCTCGGAGTACCGGTTT</td>
<td>152</td>
<td>0.8× MDE</td>
</tr>
<tr>
<td>XL2S</td>
<td>Telomeric to EST-C</td>
<td>G/A</td>
<td>AAGGCAAGGCGGAGTTTTC AGACTGAGTCAGAGGGAGAAG CGCAAGGCTGTTCTCGGAGTACCGGTTT</td>
<td>145</td>
<td>0.8× MDE</td>
</tr>
<tr>
<td>XL1S</td>
<td>Telomeric to EST-C</td>
<td>T/C</td>
<td>CAAAAGCCCGTGTGTGGTCTAC GGTTGAGACAGAGGGACTGCTGTG</td>
<td>166</td>
<td>0.8× MDE</td>
</tr>
<tr>
<td>CCS</td>
<td>EST-C</td>
<td>G/A</td>
<td>CATCGGCAACATGAGGATACG CAGCGACTGTCAGGTGGAGAG</td>
<td>145</td>
<td>1× MDE</td>
</tr>
<tr>
<td>TG2S</td>
<td>T64224</td>
<td>C/T</td>
<td>CAGAACACGACCATCAGAG GGATCCTGTTGATGGGACAG</td>
<td>187</td>
<td>1× MDE</td>
</tr>
</tbody>
</table>

Results

Genetic Map of the CHH Region

To refine the genetic map of the CHH region in chromosome 9p13 beyond our previous map (Vakkilainen et al., 1999), we developed new polymorphic markers (Table 1). Haplotypes were constructed of 116 affected chromosomes from 16 multiplex and 42 uniplex Finnish CHH families using 23 polymorphic markers. Among the 116 haplotypes, 96 (83%) shared alleles with the presumptive ancestral haplotype, suggestive of a prevalent shared founder mutation. A graphic representation of those 78 haplotypes in which all alleles could be unequivocally assigned to the 23 markers is shown in Figure 1A. The “ancestral” haplotype shown uppermost was observed in 18 chromosomes, whereas all other chromosomes showed a reduction in the length of the shared haplotype as evidence of historical recombinations. These historical recombinations allowed us to tentatively narrow the critical CHH region to the interval between the TESK1 A/G and D4S markers. This region was later shown to represent 145 kilobases of DNA.

The degree of linkage disequilibrium between markers and the disease phenotype can give a hint about the precise location of the sought gene (Hästbacka et al., 1992) and has proven a powerful tool in the positional cloning of numerous genes in the Finnish population (de la Chapelle and Wright, 1998). The higher the P_{\text{excess}} value, the stronger is the linkage disequilibrium and the closer is the marker to the disease gene (Lehesjoki et al., 1993). Within the above critical region, P_{\text{excess}} varied between 0.70 and 0.92 being highest at the adjacent markers TP2A and TA13S (Figure 1A).

Genomic and Transcript Map of the CHH Region

The CHH critical region as defined above was contained in three overlapping BAC clones, 468J10, 199G9, and 497J14 as determined previously (Figure 2 in Vakkilainen et al., 1999). The region was known to contain several genes and transcripts; however, in an effort to study all genes, we shotgun-sequenced the three BAC clones and assembled the middle part of the sequence into three contigs, 71.6 kb, 187.6 kb, and 122.7 kb in length (accession nos. AF334828 to AF334830). The 145 kb CHH critical region was fully covered by the largest contig and contained at least 11 genes as shown in Figure 1B.

Mutational Analysis in CHH Patients

Using available bioinformatics tools and existing databases, we mapped and assembled 11 genes in the critical region that we had sequenced (Figure 1B). We analyzed all 11 genes by a combination of reverse transcriptase PCR cDNA sequencing, single-strand conformational polymorphism (SSCP), and direct genomic PCR sequencing in a panel of 7 CHH patients and one noncarrier control. No potentially disease-causing mutations were found in the coding regions of TESK1, CD72, SIT, AK001187, MN/CA9, TPM2, TLN, LZIP, AAF53384 homolog, and KIAA0258 genes (data not shown). However, the finding of an insertion in the RMRP gene in one CHH patient focused our attention on RMRP.

Mutations in the RMRP Gene

The primary transcript of RMRP (accession no. M29916) comprises 267 nucleotides and is present in the genome as a single copy (Chang and Clayton, 1989). RMRP is transcribed by RNA polymerase III, which is active within
RNase MRP RNA Mutations in Cartilage-Hair Hypoplasia

Figure 1. Genetic, Physical, and Transcript Map of the CHH Region

(A) Haplotypes of 78 affected chromosomes from Finnish CHH patients are shown. Alleles of the polymorphic markers are arbitrarily numbered. The uppermost haplotype encountered in 18 chromosomes is considered “ancestral.” Portions of this haplotype that are shared on the remaining 60 chromosomes are shown in blue. The region shared by all haplotypes extends between markers TESK1 A/G and D4S and is depicted as the “CHH region.” The telomere is to the left. On top are shown the P<sub>assoc</sub> values depicting the degree of linkage disequilibrium between each marker and the disease phenotype. A peak at markers TP2A and TA13S is seen. An arrow indicates the location of the RMRP gene.

(B) Shown here is the middle region of the genetic map depicted in (A). Above the line are the 9 polymorphic markers; below the line are the transcripts. Among the genes in the CHH critical region the ones that were mapped there before the genomic sequence was obtained are marked in yellow, whereas the ones marked in blue were localized based on the sequence we generated. RMRP is depicted in red. In addition to these genes, exon prediction and homologies to human and mouse coding sequences suggested one novel gene immediately centromeric to AK001187 (MG homolog). A promoter region near the 3’ end of TLN and several non-TLN ESTs were found to overlap the 3’ end of TLN. These findings may suggest an unknown gene overlapping TLN (TU region). After repeats were masked, exons of the putative genes were searched using FGENES and GRAIL-1.3 programs. Four exons were predicted for the MG homolog whereas three exons were predicted for the TU region. Using reverse transcription PCR, we determined that AK001187 and MG homolog belong to the same gene.

the nucleoplasm (Paule and White, 2000). The RMRP promoter is a type III promoter lacking intragenic promoter elements (Murphy et al., 1987; Paule and White, 2000). A TATA box is located between −33 and −25, and additional type III promoter elements are found between −67 and −57 (PSE element), −215 and −208 (octamer element), and between −231 and −225 (SP1 binding site) (Yuan and Reddy, 1991). We PCR amplified the entire ~1 kb RMRP region in four overlapping fragments, and searched for sequence variants by direct sequencing.

The mutations are of two distinct types. The first category consists of insertions or duplications between 6 and 30 nucleotides long residing in the region between the TATA box and the transcription initiation site. As shown below, these mutations interfere with the transcription of RMRP. The second category consists of single nucleotide substitutions and other changes involving at most two nucleotides. These reside in highly conserved residues of the transcribed sequence. Table 2 summarizes the mutations found in those CHH patients and families in which a mutation of both alleles has been identified so far. Figure 2 summarizes the location of these mutations.

Of note is that while the insertion mutations are of somewhat different length and location, they all result in a lengthening of the important distance between the TATA box and the transcription initiation site. Regarding the 4 different mutations in the transcribed sequence, we note that all are located at sites that are conserved in human, cattle, mouse, rat, and some even in Xenopus, Arabidopsis, or Saccharomyces suggesting that they
Figure 2. Localization of the RMRP Mutations in CHH Patients

Depicted in the upper part is the RNase MRP RNA gene as published by Topper and Clayton (1990). Shown is the TATA box at position -233 to -225 and the transcription initiation site (represented by +1). Depicted in the box is the transcribed region of the RNase MRP gene. All mutations listed in Table 2 are shown. The lower part depicts the secondary structure of the RNase MRP RNA shown as suggested by Schmitt et al. (1993). The mutations are shown in red.

are functionally important (Schmitt et al., 1993; Sbisa et al., 1996). Moreover, nucleotides 98 and 262 reside in positions predicted to show double-stranded structure (Schmitt et al., 1993) whereas position 98 can be important for RMP40 (recently shown to be identical to Rrp 38; van Eenennaam et al., 2000) protein binding (Pluk et al., 1999). These features suggest that the mutations are extremely likely to cause functional disturbances.

Among 70 CHH patients that have been studied for mutations so far, 55 harbor a mutation in both alleles of the RMRP gene. In all instances where samples from several family members were available, the mutations cosegregated with the disease phenotype assuming recessive inheritance. In 13 families with more than one affected child, all affected individuals had identical mutations. All 26 parents studied so far were heterozygous for one of the mutations found in the affected child(ren). Among 6 unaffected siblings of affected individuals either heterozygosity for a mutation (n = 1) or homozygosity for the wild type sequence (n = 5) was seen. Notably, in Finnish patients and parents the chromosomes carrying the main 70A—G mutation all had the main “ancestral” haplotype or modifications thereof (n = 110). All 6 chromosomes carrying the 262G—T mutation shared a haplotype that differed from the main one, and both chromosomes carrying the 10 bp duplication had another, “private” haplotype.

In searching for these mutations in control individuals comprising 120 anonymous blood donors from Finland, a panel of 61 non-Finnish controls representing various nationalities, and 99 Centre d’Etude du Polymorphismes Humain (CEPH; http://www.cephb.fr) grandparents were screened. A single case of a control sample with one of these mutations was detected in Finland; a blood donor was heterozygous for 70A—G. As the carrier frequency of CHH in Finland is of the order of 1:76 (Mäkitie, 1992), this finding was expected. Among 160 non-Finnish controls, it was found in two samples. These were grandpar-
Table 2. Mutations in the RMRP Gene in CHH Patients

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Nationality</th>
<th>Mutation in the Paternal Allele</th>
<th>Mutation in the Maternal Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Finnish</td>
<td>70A→G</td>
<td>70A→G</td>
</tr>
<tr>
<td>2*</td>
<td>Finnish</td>
<td>(footnote c)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Finnish</td>
<td>70A→G</td>
<td>262G→T</td>
</tr>
<tr>
<td>4</td>
<td>Finnish</td>
<td>262G→T</td>
<td>70A→G</td>
</tr>
<tr>
<td>5</td>
<td>Finnish</td>
<td>70A→G</td>
<td>dupTACTCTGTGA at -13′</td>
</tr>
<tr>
<td>6</td>
<td>Finnish</td>
<td>dupTACTCTGTGA at -13′</td>
<td>70A→G</td>
</tr>
<tr>
<td>7*</td>
<td>Swiss</td>
<td>Two times dupACTCTGTGAAGC at -10′</td>
<td>98dupTG′</td>
</tr>
<tr>
<td>8*</td>
<td>German</td>
<td>70A→G</td>
<td>insCCTGAG at -6′</td>
</tr>
<tr>
<td>9</td>
<td>Canadian</td>
<td>98dupTG′</td>
<td>70A→G</td>
</tr>
<tr>
<td>10</td>
<td>English</td>
<td>dupTCTGTGAAAGCTGAGGAC at -3′</td>
<td>193G→A</td>
</tr>
</tbody>
</table>

* Patients 1, 2, 7, and 8 refer to the ones described in Figures 3 and 4.

1 Homozygosity for the 70A→G mutation has been diagnosed in a total of 42 Finnish CHH families, including 12 with more than one affected child and 30 with a single affected child. All of these carry modifications of the “ancestral” haplotype described in Figure 1. The 70A→G mutation has been detected in heterozygosity in 1 Finnish control sample out of 120 and in 2 out of 160 non-Finnish control samples.

3 This patient has uniparental disomy for chromosome 9 with two copies of the maternal, mutation-carrying chromosome, and no paternal chromosome 9. The patient has been described in detail (patient A in Sulisalo et al., 1997).

4 The 262G→T mutation has been found in compound heterozygosity (with 70A→G) in a total of 6 Finnish CHH families, one of which has two affected children. The chromosomes carrying 262G→T share a haplotype that differs from that of the main “ancestral” one. The 262G→T mutation has not been detected in any of 120 Finnish and 160 non-Finnish control individuals.

5 The 10-nucleotide TACTCTGTGA duplication at position -13 occurs in heterozygosity in two Finnish families (patients 5 and 6). The chromosomes carrying this mutation share a haplotype that differs from the “ancestral” one. This and all other duplication-insertions have been searched for in 120 Finnish and 160 non-Finnish controls and not found.

6 None of these mutations have been found in 120 Finnish and 160 non-Finnish controls.

7 The 193 G→A mutation has not been found in any of 112 Finnish and 93 non-Finnish controls.

RNase MRP RNA was found in all CHH patients. Using the level of expression of U3 snoRNA and U1 snRNA as references, RNase MRP RNA was less abundantly present in patients 7 and 8 as compared to control cells. Quantitative analysis using a phosphorimager showed that the level of expression of RNase MRP RNA in patients 7 and 8 was reduced to 35%–40% of the level of expression in control cells. By contrast, MRP RNA was expressed in patients 1 and 2 at a level comparable to that in the controls. In conclusion, these results suggest that the level of expression of RNase MRP RNA is reduced in the CHH patients with 5′ region insertions but not in CHH patients with merely intragenic mutations.

The Reduced Level of RNase MRP RNA Is Caused by Silencing of One RMRP Allele

Next we investigated whether the reduced expression seen in patients 7 and 8 was due to the silencing of one allele or a lower activity of both alleles. Both patients are compound heterozygous for mutations (Table 2). In patient 7, the transcript was noted to be slightly larger than in the other CHH patients and the controls, as seen in lane 3 of Figure 3. As the slight increase in transcript size suggested an addition of ~2 nucleotides, we hypothesized that the transcript emanated from the maternal allele that has a 2 bp duplication at position 98 (Table 2). To further study this phenomenon, we reverse transcribed RNA from patients 7 and 8 and a healthy control and then cloned and sequenced the cDNAs.

The sequence of RMRP RNA in control cells was identical to that published by Topper and Clayton (1990) (accession no. M29916). In patient 7, all 10 clones analyzed showed the duplication of TG at position 98, while in patient 8, all 10 clones sequenced showed the 70A→G mutation. Thus, in these two patients, the alleles with
Discussion

As shown here, mutations in RMRP can lead to CHH. Genetic evidence suggests the following model for the effect of RMRP mutations. Duplications and insertions in the region between the TATA box and the point of transcription initiation cause a lengthening of this interval and, at least in two cases, silencing of the corresponding RMRP allele. These we may call “null” promoter mutations. By contrast, mutations in the transcribed part of the gene do not cause inhibition of transcription, and may well be “leaky.” These assumptions are based on the fact that no patients have been found that are homozygous for the putative null mutations, and therefore this condition might be lethal as could be deduced from studies in yeast (Schmitt and Clayton, 1992). By contrast, patients who are homozygous “leaky” and others who are heterozygous do occur. It might not be surprising if CHH patients with a leaky/leaky genotype were less severely affected than patients who are null/leaky. Both mild and severe cases occur among the numerous patients who are homozygous for the main 70A→G mutation (‘leaky/leaky’). However, it is too early to firmly assess any genotype–phenotype correlations. Indeed, we (Maakitie and Kaitila, 1993) and others (McKusick et al., 1965) are impressed with the great phenotypic variability seen even within sibships. This suggests that other factors than (or in addition to), the RMRP mutation status contribute to the phenotype. These questions may best be addressed by studying experimental mutations in animal models.

The first description of CHH by Victor McKusick et al. (1965) established its major characteristics: recessive inheritance of a condition characterized by disproportionate, short-limbed dwarfism associated with numerous other manifestations. The multi-organ, multi-tissue manifestations of CHH, including cancer stimulated speculations about the nature of the mutated gene (Lux et al., 1970; Virolainen et al., 1978; Pierce and Polmar, 1982; Mäkitie, 1992). In the largest series of CHH patients studied for cancer, a significant excess of cancer was seen, and it was mainly attributable to non-Hodgkin’s lymphoma (Mäkitie et al., 1999). Our results are compatible with the hypothesis that the basic defect may involve many cellular functions. The RNase MRP complex is one of a large population of small nucleolar ribonucleoproteins (reviewed by Tollervey and Kiss, 1997). In yeast the RNase MRP complex cleaves pre-rRNA (Chu et al., 1994). The RNA gene that is mutated in CHH was first described as a nuclear gene whose RNA product contributes to the endonuclease activity needed for mitochondrial DNA replication (Chang and Clayton, 1987a, 1987b, 1989). By immunoprecipitation we showed that none of the mutations analyzed abolish the association of these four protein subunits with the MRP RNA.

5′ region insertions were not expressed. Taken together with the fact that total expression levels in these two patients were only 35%–40% of the controls (Figure 3), it follows that only the alleles with intragenic mutations were expressed.

RNase MRP Protein Subunits Associate with RNase MRP RNA in CHH Patients

To study protein binding to MRP RNA, we performed immunoprecipitations on cell extracts derived from the four CHH patients described in the preceding paragraph, and two controls.

As shown in Figure 4, hPop1 and hPop4 were clearly associated with the RNase MRP complex in patients 1, 2, 7, and 8. Similar experiments were done using antibodies against the Rpp30 and Rpp38 protein subunits (Eder et al., 1997) yielding similar results (data not shown). In summary these immunoprecipitation data suggest that none of the mutations analyzed abolish the association of these four protein subunits with the MRP RNA.

Figure 4. Association of hPop1 and hPop4 Protein Subunits with the RNase MRP Complex

(A) Immunoprecipitation with anti-hPop1 and anti-hPop4 antibodies on extract of two CHH patients, a control cell line and HeLa cell extract. The control antibody is preimmune serum. Lanes 1–4 represent 10% input fraction, lanes 5–8 the coprecipitating MRP RNA using anti-hPop1 antibodies, and lanes 9–12 the coprecipitating MRP RNA using anti-hPop4 antibodies. The signal with preimmune serum in HeLa cells is background due to overloading of cell extract.

(B) Immunoprecipitation performed using anti-hPop1 antibodies on extract of CHH patients 7 and 8 and control cells. Lanes 1–3 represent the 10% input fraction, lanes 4–6 the coprecipitating MRP RNA with antibodies. (C) Immunoprecipitation performed using anti-hPop4 antibodies on extract of CHH patients 7 and 8 and control cells. Lanes 1, 4, and 7 represent the 10% input fraction, lanes 2, 5, and 8 the coprecipitating MRP RNA with antibodies.
Thus a clear-cut disturbance of one of the two known functions of RNase MRP has not yet been documented in CHH cells. It may even be that the RNA product of RMRP has other functions in addition to the ones characterized so far, and that the mutations found in CHH patients disrupt these. The fact that mutations in the transcript were found only in the highly conserved positions may eventually yield clues in this regard. In analogy with other small nuclear and small nucleolar ribonucleoprotein particles, these residues might be located in regions that are involved in substrate RNA recognition by direct base pairing interactions (Tollervey and Kiss, 1997). Alternatively, they might be part of the catalytic center of the enzyme, a possibility supported by the structural homology with RNase P, one of the first RNAs for which a catalytic activity has been identified (Guerrero Takada et al., 1983). Interestingly phylogenetic comparison of RNase MRP and RNase P RNA between human and E. coli supports this idea. The sequence element 68-GGAA-71 in human RNase MRP RNA is found to be fully conserved (Forster and Altman, 1990). The functional significance of these residues is suggested by recent studies on the E. coli RNase P complex, in which the two adenosine residues at comparable positions (70 and 71) in RNase MRP RNA are important for substrate binding and catalysis (reviewed by Kurz and Fierke, 2000). Thus, it is tempting to speculate that the A-G substitution at position 70 found in the major haplotype of CHH interferes with the optimal activity of the RNase MRP enzyme.

An unknown function has been previously alluded to by Tollervey and Kiss (1997) who suggested the existence of yet undetected additional substrates for RNase MRP. As reviewed by van Eenennaam et al. (2000), a function in cell cycle control is possible. In yeast cells having RMRP mutations, defects in plasmid segregation believed to be caused by telophase arrest have been documented (Cai et al., 1999). Disturbances in the mitotic process would seem to fit the T cell deficiency described in CHH patients. The immune deficiency, together with the hypoplastic anemia, and the failure of skeletal growth in CHH, would seem to be logical effects of, for example, mitotic arrest.

Experimental Procedures

Genetic Mapping of the CHH Region

Only Finnish CHH patients with a carefully verified diagnosis were studied for the purpose of refining the genetic map. There were 16 families with more than one affected individual (multiplex), and 42 families with one affected individual (uniplex). Several individuals per family were genotyped for 23 polymorphic markers. From each family only 2 affected haplotypes (one maternal, one paternal) were considered in the analysis. Haplotypes were constructed manually. The presumptive ancestral founding haplotype was reconstructed assuming the minimum number of historical recombinations. A measure of linkage disequilibrium between a marker and the disease phenotype \( P_{\text{disequilibrium}} \) is calculated using the formula

\[
P_{\text{disequilibrium}} = \frac{(P_{\text{disequilibrium}} - P_{\text{null}})(1 - P_{\text{null}})}{\text{number of recombinations}}
\]

where \( P_{\text{disequilibrium}} \) is the frequency of the allele in affected chromosomes and \( P_{\text{null}} \) is the frequency of the allele in unaffected chromosomes (Lehesjoki et al., 1993).

For these calculations most of the 116 affected haplotypes were used that were judged to descend from the putative shared major founder mutation. The corresponding normal haplotypes (\( n > 100 \) at each marker) were taken from the unaffected maternal and paternal chromosomes. The number of haplotypes (affected and normal) varied slightly between markers because of occasional uncertainty in allele assignment.

Analysis of Polymorphic Markers

Most of the novel polymorphic markers were found during SSCP analyses or sequencing of candidate genes in patient samples. Primers for the SSCP markers and the genomic 13 nt deletion (Del13) were designed using the Primer 3 program at the Whitehead Institute for Biomedical Research/MIT Center for Genome Research at http://www.genome.wi.mit.edu (Table 1). Primers 5'-TCCCTGGAAGAG TACGAAA-3' and 5'-GCGGAGAAGAGTGCTCT-3' were used for D9S1878 (http://www.ceph.fr/mail.html). Primers for the other polymorphisms were listed in Table 2 in Vakkilainen et al. (1999). For the SSCP analyses, the PCR products were loaded on MDE (FMIC BioProducts) gels and run overnight at room temperature (Table 1). Markers D9S1878, D9S163, D9S1804, 99AC, 89AC, and AC1 were separated in 6% Sequapel (National Diagnostics) gels at 50°C. Bands on the polyacrylamide gels were visualized by silver staining as described in detail by Pein et al. (1999).

Genomic Sequencing of the CHH Region

Three BAC clones (497J14, 199G9, and 468J10) from the previously assembled contig (Vakkilainen et al., 1999) covering the CHH critical region were shotgun sequenced. Libraries were prepared in pCR-BluntII-Topo vector (Invitrogen) using the GATC GmbH service (Konstanz, Germany). Forty 96-well plates were sequenced, in both directions, using the BigDye Terminator AmpliTaq FS Cycle Sequencing Kit (PE Applied Biosystems). Cycle Sequencing was performed using the PE9700 Thermal Cycler (Perkin Elmer). The cycling protocol was 30 cycles of 95°C for 30 s, 50°C for 20 s, and 60°C for 4 min. Cycling was performed using 10 \( \mu l \) reactions and reactions were purified by filtration plate. Custom vector primers TOP20 5'-AGATGCATGTCGCCCGG-3' and TOP01 5'-TCCGATGCCACTAGTAAAGC-3' designed by GATC GmbH, were used. Sequences were processed through a series of programs in UNIX.

After quality assessment using Phred, 72% of subclone sequences were passed for use in assembling contigs using Phrap. Gap filling was performed using a combination of primer walking from the contig ends and PCR across the gaps. Primer sequences and PCR conditions are available on request. Finishing was facilitated by using MIT's Finish program (http://www.genome.wi.mit.edu). The genomic sequence of the 187.6 kb CHH region was analyzed using the Genotator program package.

Patients and Controls

Mutational analyses have so far been done on 51 CHH families from Finland and 4 families from other countries. With few exceptions both parents and all affected children have been studied. In addition a small number (\( n \sim 7 \)) of unaffected children have also been studied. The diagnostic criteria were as outlined previously (Mäkitie and Kaitila, 1993). To search for the observed sequence variants in controls, 120 anonymous blood donors from Finland, a panel of 61 Finnish CHH patients with a carefully verified diagnosis were documented (Cai et al., 1999). Disturbances in the microsatellite analyses or sequencing of candidate genes in patient samples.

Mutation Detection in RMRP

The RMRP gene (accession no. M29916) was sequenced in four PCR fragments RM1–RM4. The primers were RM1F, 5'-GCTGGAC CCTATCGGGAGG-3'; RM1R, 5'-GACACCCCGTACGCTAGTAC-3'; RM2F, 5'-GGAGGATACAGCGGCGGTC-3'; RM2R, 5'-GGACGATGCTACAGCAGGGC-3'; RM3F, 5'-GCGGACGACCATATTTCGACTAAGG-3'; RM3R, 5'-GCGGACTTTGGAGTGGGAAG-3'; RM4F, 5'-AGAGATGTCGCCAGTCTAC-3'; RM4R, 5'-GTCTCTAGACAGGCGAAGAAC-3'. Primers for RM3 fragment inside RMRP were 5'-GTTA GAAAGTTATGCCCGAAAAC-3'; RM3F, 5'-GCTGGATACAGGCGAGTCAG-3'. After quality assessment using Phred, 72% of subclone sequences were passed for use in assembling contigs using Phrap. Gap filling was performed using a combination of primer walking from the contig ends and PCR across the gaps. Primer sequences and PCR conditions are available on request. Finishing was facilitated by using MIT’s Finish program (http://www.genome.wi.mit.edu). The genomic sequence of the 187.6 kb CHH region was analyzed using the Genotator program package.

For these calculations most of the 116 affected haplotypes were used that were judged to descend from the putative shared major
searched for within 1.5 kb from the M29916 sequence by running PCR fragments in 5% Sequagel (National Diagnostics) and Long-Ranger (FMC BioProducts) gels at 50°C. The primer sequences and PCR conditions are available on request from the authors.

Extraction and Analysis of Total RNA
Lymphoblasts (Control-L, patient 1 and patient 2) and fibroblast (Control-F, patient 7 and patient 8) cells were pelleted and total RNA isolated using Trizol (Gibco-BRL) as suggested by the manufacturer. RNAs were resolved on 5% denaturing polyacrylamide gels and blotted to Hybond-N+ membranes (Amersham). Northern blot hybridizations with riboprobes specific for human RNase MR, U3 snoRNA, and U1 snRNA were performed as previously described (Verheijen et al., 1994). Quantitative analysis of the levels of transcription was done with a Phosphorimager (Biorad).

Synthesis and Analysis of cDNA

Acknowledgments
We dedicate this paper to Dr. Victor A. McKusick.

References


