The Imprinted NPAP1 Gene in the Prader–Willi Syndrome Region Belongs to a POM121-Related Family of Retrogenes

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Abstract

We have recently shown that the human Nuclear pore-associated protein (NPAP1)/C15orf2 gene encodes a nuclear pore-associated protein. This gene is one of several paternally expressed imprinted genes in the genomic region 15q11q13. Because the Prader–Willi syndrome is known to be caused by the loss of function of paternally expressed genes in 15q11q13, a phenotypic contribution of NPAP1 cannot be excluded. NPAP1 appears to be under strong positive Darwinian selection in primates, suggesting an important function in primate biology. Interestingly, however, in contrast to all other protein-coding genes in 15q11q13, NPAP1 has no ortholog in the mouse. Our investigation of the evolutionary origin of NPAP1 showed that the gene is specific to primate species and absent from the 15q11q13-orthologous regions in all nonprimate mammals. However, we identified a group of paralogous genes, which we call NPAP1L, in all placental mammals except rodents. Phylogenetic analysis revealed that NPAP1, NPAP1L, and another group of genes (UPF0607), which is also restricted to primates, are closely related to the vertebrate transmembrane nucleoporin gene POM121, although they lack the transmembrane domain. These three newly identified groups of genes all lack conserved introns, and hence, are likely retrogenes. We hypothesize that, in the common ancestor of placentals, the POM121 gene retrotransposed and gave rise to an NPAP1-ancestral retrogene NPAP1L/NPAP1/UPF0607. Our results suggest that the nuclear pore-associated gene NPAP1 originates from the vertebrate nucleoporin gene POM121 and—after several steps of retrotransposition and duplication—has been subjected to genomic imprinting and positive selection after integration into the imprinted SNRPN-UBE3A chromosomal domain.

Key words: C15orf2, NPAP1L, UPF0607, imprinting, nucleoporin, nuclear pore complex.

Background

Genomic imprinting is a process that regulates the expression of certain genes in a parent-of-origin-dependent manner and has evolved independently in plants and mammals (Feil and Berger 2007). Genes regulated by this epigenetic mechanism are thus expressed either from the maternal chromosome or the paternal chromosome only. Most imprinted genes are organized in clusters, which are regulated by imprinting control regions (ICRs) that regulate the monoallelic expression of the genes in cis (Reik and Maher 1997; Ferguson-Smith 2011).

The human genomic region 15q11q13 contains a cluster of imprinted genes with several paternally only expressed genes and one maternally only expressed gene (fig. 1). It is regulated by an ICR that includes the promoter of the SNRPN gene (Buiting et al. 1995; Saitoh et al. 1996; Ohta et al. 1999; Horsthemke and Buiting 2008). Loss of function of the paternally expressed genes in this region, most commonly arising through a ~6 Mb deletion on the paternally inherited chromosome, leads to Prader–Willi syndrome with neonatal muscular hypotonia and failure to thrive, childhood-onward hyperphagia and obesity, and mild-to-moderate intellectual disability (Cassidy 1997; Butler and Palmer 1983; Buiting 2010).

We have recently shown that the paternally expressed gene C15orf2 encodes a nucleoporin complex (NPC) associated protein, and it was therefore renamed to nuclear...
pore-associated protein 1 (NPAP1) (Neumann et al. 2012). The exact function of the protein is not known, but we suspect a brain-specific NPC-related function because the protein is expressed in several human brain regions (Wawrzik et al. 2010). Several studies showed that NPAP1 underwent strong positive Darwinian selection in the primate lineage (Nielsen et al. 2005; Kosiol et al. 2008; Wawrzik et al. 2010).

The imprinted domain in 15q11q13 has assembled relatively recently during mammalian evolution from unlinked and nonimprinted components (Rapkins et al. 2006). The region evolved its imprinted regulation after fusion of SNRPN and UBE3A, most probably by retrotransposition (Gray et al. 2000; Chai et al. 2001; Rapkins et al. 2006). It was hypothesized that at least some of the retrogenes integrated into the region after the evolution of imprinting in 15q11q13 and acquired their imprinted regulation subsequently (Chai et al. 2001; Rapkins et al. 2006). In the well-described murine orthologous region, two rodent-specific imprinted genes, Frat3/Peg12 and Atp5l-ps1, have been identified, suggesting that the process of gene acquisition is still ongoing and leads to divergent imprinted gene sets in the primate and rodent lineage (Chai et al. 2001). Here, we report that NPAP1 is a primate-specific gene that entered the imprinted region 15q11q13 and acquired their imprinted regulation subsequently (Chai et al. 2001; Rapkins et al. 2006).

Materials and Methods

Bioinformatic Tools

NPAP1 homologous gene sequences were found using the “orthologs” list in the Ensembl database (www.ensembl.org, last accessed January 20, 2014) or using the Blast-like alignment tool (BLAT) in the University of California, Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start, last accessed January 20, 2014). Gene and protein alignments were produced using the ClustalW multiple alignment tool included in the Geneious Pro 5.6 package (Biomatters Ltd, Auckland, New Zealand) with standard settings. The prediction of intronless ORFs was also carried out with the ClustalW multiple alignment tool included in the Geneious Pro 5.6 package (Biomatters Ltd, Auckland, New Zealand) with standard settings. The prediction of intronless ORFs was also carried out with the ClustalW multiple alignment tool included in the Geneious Pro 5.6 package (Biomatters Ltd, Auckland, New Zealand) with standard settings. The prediction of intronless ORFs was also carried out with the ClustalW multiple alignment tool included in the Geneious Pro 5.6 package (Biomatters Ltd, Auckland, New Zealand) with standard settings.

Sequence Analyses

Marmoset NPAP1 sequencing was performed with Big Dye Terminators (BigDye Terminator v1.1 Cycle Sequencing Kit, Life Technologies, Darmstadt, Germany) and the cycle sequencing procedure. Products were analyzed with an ABI 31000 Genetic Analyzer and Sequencing Analysis software (Life Technologies). The following primers were used: Marmoset_NPAP1_fw1: AAACACCCCAGCTCCGTGAGGA; Marmoset_NPAP1_rev1: GGATGGGCTGGGAAGTTGTGGC; Marmoset_NPAP1_fw2: CACAACAGGCCCTGCAAAAGGA; Marmoset_NPAP1_rev2: CCCCATGTAAAACGGGAGGCAC;
Marmoset_NPAP1_fw3: ATCCAATTCTGGGCTTTG; Marmoset_NPAP1_rev4: TCCAAGGTGCCAGGGCTTC.

Expression Analyses
For expression analysis of NPAP1, we used a customized panel of cDNAs from bovine tissues (caudate nucleus, cerebellum, cerebral cortex, hippocampus, hypothalamus, kidney, liver, placenta, skeletal muscle, testis) and DNA from bovine testis as a control (Zyagen, San Diego, USA). Polymerase chain reaction (PCR) assays were designed such that they spanned introns from the Ensembl gene prediction. For intron 1 (1,067 bp), we used the primers Cow-NPAP1L-in1-fw: TAA CTATCCCTTTGACTCCCAGA and Cow-NPAP1L-in1-rev2: CTG GAGCATAAGATAACTGCCAA. For intron 2 (17 bp), we used the primers Cow-NPAP1L-in2-fw: CAAGCCTCAAATTTTG CCTG and Cow-NPAP1L-in2-rev: TGGGAAACCTGACCTT CTTG. Representative products were sequenced using Applied Biosystems BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) and an ABI PRISM 3100 Genetic Analyzer (Life Technologies). The control PCR on bovine ATCB was performed with primers Fw2-beta-Actin-Cow: GGCACCCAGCACAATGAAGA and Rev2-beta-Actin-Cow: CGACTGCTGTACCTCAGCG.

Phylogenetic Analyses
BlastP searches at NCBI (http://blast.ncbi.nlm.nih.gov, last accessed January 20, 2014) were performed using the peptide sequence of NPAP1 as query. The ML tree based on the JTT + Ψ amino acid substitution model was calculated using the MEGA5 program (Tamura et al. 2011) and the embedded alignment software MUSCLE (Edgar 2004), with a data set including all obtained sequences plus homologs identified by Ensembl.

Results and Discussion
Conservation of NPAP1 Orthologs in Primates
Ensembl (release 69; http://www.ensembl.org, last accessed January 20, 2014; [Hubbard et al. 2009]) contains NPAP1 orthologs in the chimpanzee (Pan troglodytes), gorilla (Gorilla gorilla), orangutan (Pongo abelii), and rhesus macaque (Macaca mulatta). In an effort to identify additional NPAP1 orthologs in the 15q11q13 orthologous regions of other primate species, we searched the UCSC genome browser (http://genome.ucsc.edu, last accessed January 20, 2014; [Kent 2002]) using the BLAT Kent et al. [2002]) with the human gene sequence as query. This query resulted in the identification of NPAP1 orthologs in the gibbon (Nomascus leucogenys), the squirrel monkey (Saimiri boliviensis), and the marmoset (Callithrix jacchus). But in the genome of the bushbaby (Otodelmur garnetti), a member of the more distantly related suborder of Strepsirrhini (fig. 2), we could not find an NPAP1-homologous sequence in the 15q11q13-orthologous region. The genomes of baboon (Papio anubis), tarsier (Tarsius syrichta), and mouse lemur (Microcebus murinus) are not sufficiently assembled to determine syntenic relationships.

Of all analyzed primates, the marmoset and the squirrel monkey, members of the Haplorhini parvorder Platyrhini, were the most distantly related species that already have orthologs of NPAP1 (fig. 2). An alignment showed that the marmoset NPAP1 gene on chromosome 6 contains a ~2.5 kb gap in the part that aligns with the human open reading frame (ORF). We thus sequenced the missing part of the gene in the marmoset and obtained a complete gene sequence (supplementary table S1, Supplementary Material online), that is, 69.9% identical with the human NPAP1. In contrast to NPAP1 orthologs of Catarrhini species, the marmoset NPAP1 lacks a long intronless ORF. GENSCAN (http://genes.mit.edu/GENSCAN.html, last accessed January 20, 2014, [Burge and Karlin 1997]) predicts a 3.06 kb ORF with two small introns that starts 164 bp upstream of the human ORF and ends parallel at an apparently homologous position (supplementary table S1, Supplementary Material online). The second and third exon of the GENSCAN prediction contain six deletions when compared with the Catarrhini genomes that are multiples of 3 bp, and up to 39 bp long, so would appear to be in frame and coding. By contrast, the upstream part of the gene contains a number of small indels that are not multiples of three, suggesting that this region is not part of the ORF and might have become partially pseudogenized. The indel pattern suggests a balancing selection against frame-shifts inside the coding sequence, as 1 bp indels occur with approximately ten times higher probability than 3 bp indels (de la Chaux et al. 2007). This argues for a protein coding function of marmoset NPAP1 despite the absence of the expected intronless ORF. As NPAP1 orthologs were found in all analyzed members of Haplorhini but not in any members of Strepsirrhini (fig. 2), the gene presumably integrated into the 15q11q13 orthologous region after the two primate suborders diverged about 60–70 Ma (Springer et al. 2012).
parsimoniously, the nonprimate homologs might be of different origin. Therefore, we investigated the conservation of synteny among the Ensembl orthologs more closely. Using the UCSC genome browser, we found that only primate NPAP1 genes share synteny with UBE3A and SNRPN, whereas the homologous genes in other mammals are located between their respective orthologs of transducin-like enhancer of split (TLE) 1 and TLE4 (fig. 3). We refer to the group of orthologous genes located in this synteny group as nuclear pore-associated protein 1-like (NPAP1L). TLE1 and 4 belong to a larger region of well-conserved synteny including orthologs of the human genes GNA14, GNAQ, PSAT1, TLE4, TLE1, RASEF, and FRMD3 (centromeric to telomeric on human chr9q21). Because the complete synteny group can also be found in chicken, which does not contain NPAP1L, it is likely that NPAP1L integrated into this region as a single gene during mammalian evolution. A possible mechanism for this would be retrotransposition (Kaessmann 2010).

Ensembl (release 69) also contains genes from three additional mammalian species (cat, ENSFCAG00000010320; tree shrew, ENSTBEG00000007225; ferret, ENSMPUG00000019448) that were annotated as NPAP1. The current quality of the genome assemblies of these species does not allow us to conduct robust synteny analyses. However, cat scaffold GL897178.1 contains both TLE1 and ENSFCAG00000010320, suggesting that ENSFCAG00000010320 is another ortholog of NPAP1L. All Ensembl-annotated NPAP1L orthologs were predicted to be protein coding by the Ensembl pipeline (Potter et al. 2004).

Using the UCSC genome browser and the BLAT algorithm with human NPAP1 as query, we found NPAP1L sequences in the genomes of additional mammals, for example, horse and rabbit. We could, however, not identify significant hits in either rodents or marsupials. In the genomes of mouse and rat, TLE4 (on murine chr.19) and TLE1 (on murine chr. 4) have lost their syntenic position, creating the impression that NPAP1L might have been lost in their common ancestor during chromosomal rearrangements. In the guinea pig genome, however, the TLE4-TLE1 synteny group is found on scaffold 21 although NPAP1L was not identified, rather suggesting that NPAP1L has been lost in rodents before chromosomal rearrangements in this region. In the human genome, our search revealed two copies of NPAP1L in opposite orientations on human chromosome 9 that match Ensemble-annotated processed pseudogenes (ENSG00000238002 on the plus strand and ENSG00000236521 on the minus strand). The same arrangement of NPAP1L genes was also observed in other primate species (fig. 3). Both human NPAP1L copies contain only short ORFs, which code for peptides without significant amino acid sequence similarity to FIG. 2.—NPAP1 conservation in primates. (A) Cladogram showing the family structure of primates and the relationship of the analyzed primate species. NPAP1 orthologs were found in all analyzed members of the parvorders Platyrrhini and Catarrhini (red). (B) Detailed view of an alignment of selected primate NPAP1 orthologs. The figure shows a well-conserved region inside the human ORF (1,965–2,062 bp from NCBI reference sequence NM_018958.2) that contains one of the numerous in-frame deletions found in the marmoset sequence.
NPAP1 and NPAP1L proteins, confirming their annotation as processed pseudogenes. As is the case for the human genome, also the genomes of dog and elephant each contain two highly similar copies of NPAP1L with opposite orientations. However, in contrast to dog and elephant, the human NPAP1L copies are tail-to-tail oriented, suggesting that NPAP1L tandem-duplicated several times independently (fig. 3).

Expression Analysis of NPAP1L

As mentioned earlier, all nonprimate NPAP1L orthologs were predicted to be protein coding by Ensembl. In an effort to verify the in silico prediction in a nonprimate species, we analyzed the expression of bovine NPAP1L. To this end, we used a bovine cDNA panel (Zyagen) and two PCR assays spanning the two introns of the Ensembl-predicted gene (ENSBTAG00000046462). As we had shown before that human NPAP1 is expressed in testis and brain (Färber et al. 2000; Wawrzik et al. 2010), we included cDNA from different bovine brain regions and testis into our panel. With both PCR assays, we observed expression of bovine NPAP1L in four of five analyzed brain regions and testis into our panel. With both PCR assays, we observed expression of bovine NPAP1L in four of five analyzed brain regions: caudate nucleus, cerebellum, hippocampus, and hypothalamus. With one of the two assays, we also observed weak signals for the cerebral cortex, kidney, and testis, but we did not observe expression in liver, placenta, or skeletal muscle (data not shown). Curiously, by gel analysis and Sanger sequencing, the obtained products from cDNA did not correspond to mRNA of the expected splicing pattern but were colinear with genomic DNA.

Although our cDNA panel had been obtained from a commercial source (Zyagen) that tests for residual DNA contamination as part of their quality control procedure, the unexpected finding of colinear RNA expression prompted us to double-check for contamination with genomic DNA. To this end, we used an intron-spanning RT-PCR for the ACTB locus that gives rise to a 131-bp larger product when genomic DNA is amplified. In all cDNA samples, we only obtained the product that is expected from ACTB cDNA, making a contamination of the cDNAs with genomic DNA very unlikely. We conclude that NPAP1L is expressed in the cow, primarily in the brain, but that the splicing pattern predicted by Ensembl is not correct in any of the analyzed tissues. Alternatively, it is possible that a 2,688-bp intronless ORF is expressed in the cow and would lead to a shorter NPAP1-homologous protein of 895 amino acids. Even if the bovine NPAP1L ortholog should not be protein coding, this would not per se exclude protein-coding functions for other mammalian NPAP1L orthologs.

NPAP1 and NPAP1L Belong to a POM121-Related Gene Family

Because functional NPAP1L sequences could be found in all analyzed mammals except rodents, whereas NPAP1 is unique to primate species, we hypothesized that NPAP1 originates from the NPAP1L gene locus. In order to investigate this hypothesis in an unbiased way, we performed a BlastP (http://blast.ncbi.nlm.nih.gov, last accessed January 20, 2014) search with the human NPAP1 protein sequence as query. Sequences obtained in this search as well as Ensembl-annotated protein sequences were used for the inference of phylogenetic relationships. A maximum likelihood (ML) tree (fig. 4) was reconstructed using representatives of major tetrapod taxa. This data set consisted of NPAP1 amino acid sequences from different primate species, of a group of proteins designated as UPF0607 that are also restricted to primates, of NPAP1L
FIG. 4.—ML tree of NPAP1-homologous proteins. Bootstrap values (left) and associated support values (right) are given for all nodes that have bootstrap values above 50. The tree topology suggests that NPAP1, UPF0607, and NPAP1L are derived from the vertebrate-specific nucleoporin gene POM121. NPAP1L seems to be ancestral to the primate-specific sister genes NPAP1 and UPF0607. On the basis of branch lengths, it can be seen that the two neighboring NPAP1L copies (NPAP1La and NPAP1Lb) in dog and elephant are the result of two independent duplications.
proteins from elephant, dog, cat, pig, and cow, and of distantly related POM121 and POM121-like proteins (supplementary table S2, Supplementary Material online).

The inferred ML tree suggests that the genes NPAP1L, NPAP1, and UPF0607 form a monophyletic group that is derived from POM121 (fig. 4). The most straightforward interpretation of the tree topology and the taxonomic distribution of genes is that NPAP1L duplicated in the lineage leading to primates, resulting in an NPAP1/UPF0607 gene. Before the primate radiation, this ancestral NPAP1/UPF0607 gene duplicated again to give rise to NPAP1 and UPF0607. The primate-specific UPF0607 gene duplicated in a subset of primate species resulting in two groups of genes, namely UPF0607a and -b (see fig. 4 and supplementary table S2, Supplementary Material online). In dog and elephant, two copies of NPAP1L genes derived from recent independent duplication events are found, namely NPAP1La and -b (see fig. 4 and supplementary table S2, Supplementary Material online). In the lineage leading to primates, the ancestral NPAP1L gene pseudogenized and is therefore not included in our phylogenetic analysis. However, remnants of this gene are still identifiable in all analyzed primate species. Taken together, the tree topology supports our initial hypothesis that NPAP1 is derived from the NPAP1L gene locus and adds POM121 as a common ancestor of both groups of genes.

Although the vertebrate nucleoporin gene POM121 contains several large and highly conserved introns, all NPAP1 and UPF0607 genes are intronless. NPAP1L genes are predicted to contain small introns; however, the intronic structure is not conserved between the orthologs and presumably evolved secondarily from an intronless ancestral gene. In light of these observations, the phylogenetic analysis suggests an evolutionary scenario wherein POM121 duplicated via retrotransposition in the last common ancestor of placentals, giving rise to the intronless NPAP1L/UPAP1/UPF0607 retrogene (fig. 5). The first exon of POM121 that includes its transmembrane domain was lost during or following retrotransposition, because none of the orthologs of NPAP1L, NPAP1, or UPF0607 are predicted transmembrane proteins. The loss of 5' gene parts is characteristic of gene duplications via retrotransposition (Ding et al. 2006). More recently, before the primate radiation, this gene duplicated or retrotransposed twice more giving rise to the genes NPAP1 and UPF0607. In dog and elephant, NPAP1L was subject to independent tandem duplications, whereas it was lost in the rodent lineage (fig. 5).

Conclusions

Our results show that the imprinted, primate-specific NPAP1 gene originates from the vertebrate nucleoporin gene POM121. It is part of a so far unrecognized gene family of POM121-related retrogenes, the members of which can be considered possible candidates for mammal- and
primate-specific NPC-associated functions. Unlike POM121, the predicted proteins lack a transmembrane domain and thus appear to have functionally diverged from their ancestral protein.

**Supplementary Material**

Supplementary tables S1 and S2 are available at [Genome Biology and Evolution online](http://www.gbe.oxfordjournals.org/).

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