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Genes and Phenotypes

Mapping of a novel MEN-like syndrome locus to rat Chromosome 4

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Abstract

Multiple endocrine neoplasia-like syndrome (MENX) is a hereditary cancer syndrome in the rat characterized by inborn cataract and multiple tumors affecting the neuroendocrine system developed within the first year of life. The spectrum of affected organs is intermediate between MEN type 1 (MEN1) and MEN type 2 (MEN2) syndromes in human, but, in contrast to them, MENX is inherited in a recessive fashion. Here we report the mapping of the *MENX* locus to rat Chromosome (Chr) 4 by a genome-wide linkage analysis. This analysis was done in 41 animals obtained from a (Wistar/Nhg × SD^{we}) × SD^{we} interstrain backcross, where SD^{we} (Sprague-Dawley white eye) indicates the affected animals. The *MENX* disease locus was ultimately mapped to a ~22-cM interval on Chr 4 that includes the rat homolog of the human RET proto-oncogene. As activating point mutations of RET are known to be responsible for MEN2 in human, we analyzed several markers located in the proximity of *Ret* for linkage to the disease phenotype. Our data exclude *Ret* involvement in MENX and establish that a second gene, playing a role in endocrine tumor formation, lies within the distal part of rat Chr 4. Although heritable human endocrine tumors are quite rare, sporadic tumors of MEN-affected tissues occur at a much higher frequency, and their pathogenesis is poorly understood. The identification of the *MENX* gene should contribute to our understanding of the genetic mechanisms of neuroendocrine tissue tumorigenesis and may assist in developing new and more appropriate therapeutic strategies for these diseases.

Multiple endocrine neoplasia (MEN) is characterized by the occurrence of tumors involving two or more neuroendocrine tissues. Two major forms, referred to as MEN type 1 (MEN1) and MEN type 2 (MEN2) exist in humans and are inherited as autosomal dominant traits with high penetrance. The spectrum of affected organs differs slightly: MEN1 is characterized by the involvement of parathyroid, pancreatic islet, and anterior pituitary tissues (reviewed by Zarnegar et al. 2002), while MEN2 is characterized by medullary thyroid carcinoma (MTC) with or without phaeochromocytoma and parathyroid adenoma. There are two clinical MEN2 variants, referred to as MEN2A and MEN2B, which vary in aggressiveness of MTC and the spectrum of additional affected organs (reviewed in Hansford and Mulligan 2000).

Genetic defects underlying human MEN syndromes have been elucidated. MEN1 is caused by inactivating mutations of the Menin (MEN1) gene (Chandrasekharappa et al. 1997), whereas MEN2 arises as a result of mutations of the RET proto-oncogene (Donis-Keller et al. 1993; Mulligan et al. 1993; Hofstra et al. 1994; Mulligan et al. 1994). Menin is a putative tumor suppressor whose function has proven quite elusive, especially because it has no homology to other proteins. Recent data on Menin-interacting proteins (Poisson et al. 2003) and the generation of a mouse model for MEN1 syndrome (Crabtree et al. 2001) are helping elucidate its role in tumorigenesis and in several cellular processes, including cell growth and genomic stability. Consistent with its putative role as a tumor suppressor gene, most pathogenic MEN1 mutations so far identified correspond to a loss of function, and the tumors of MEN1 patients show loss of the wild-type allele (Poisson et al. 2003). RET encodes a receptor tyrosine kinase that is a member of the cadherin superfamily and transduces signals for cell growth and differentiation (Takahashi et al. 1988;

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Schneider 1992; Iwamoto et al. 1993). RET is mainly expressed in neural and urogenital precursor cells and plays a critical role in neural crest development, renal ontogenesis, and differentiation of spermatogonia (reviewed in Alberti et al. 2003). In MEN2 RET undergoes oncogenic activation via point mutations where the position of the mutated base is strongly associated with disease phenotype (Santoro et al. 2002).

We recently identified a novel variant of MEN in the rat (Fritz et al. 2002). Although this syndrome shows a phenotypic overlap with both MEN1 and MEN2, it is characterized by a unique combination of affected organs, namely, bilateral pheochromocytoma and parathyroid adenoma in conjunction with multifocal thyroid C-cell hyperplasia and multiple paragangliomas. Pituitary tumors associated with MEN-like Syndrome (MENX) in the rat have been shown to be multifocal (B. Palme, M. Atkinson unpublished). In addition, affected animals develop macroscopically visible bilateral cataracts in the first few weeks of life. Importantly, in contrast to the human syndromes, MENX is inherited in a recessive fashion (Fritz et al. 2002).

We report here the mapping of the locus responsible for MENX to a ~22 cM region on the distal part of Chr 4, and the exclusion of the rat *RET* homolog as the candidate gene for the disease.

Materials and methods

Animals, crosses, and phenotypes. The MENX phenotype was first identified in a Sprague Dawley (SD) rat breeding colony. The founder rats were imported from the University Eye Clinic (Munich) into the GSF animal facility in 1994 and were subsequently maintained as an inbred strain by matings between affected and unaffected littermates. As previously reported (Fritz et al. 2002), homozygous animals develop bilateral cataracts early in life, and this phenotype was used as a surrogate marker to separate unaffected from affected littermates; the latter animals have been referred to as SD^{we} (white eye; Fritz et al. 2002).

Inbred Wistar (WIST/Nhg) rats for mapping experiments were obtained from the GSF breeding stock. The WIST/Nhg strain has been deposited in the Rat Genome Database (http://rgd.mcw.edu/tools/strains), and its origin was already described (Murray et al. 1985). Animals were housed according to national laboratory animal welfare regulations. An association study was performed on a panel of 41 animals from a (WIST/Nhg × SD^{we}) × SD^{we} backcross. Among those animals, 20 were affected and 21 unaffected, with an equal distribution of the sexes.

Molecular genetic analyses. DNA was extracted from tail tips with the DNeasy Qiagen kit (Hilden, Germany). A set of 100 microsatellite markers were selected from the database based on the presumptive length variation difference between SD and WIST/Nhg strains. Sixty of those markers proved informative in our animals and were used for the initial genome-wide association screening. Additional markers located on Chr 4 were subsequently analyzed to fine-map the *MENX* locus. Animals were screened for homozygosity (affected) or heterozygosity (unaffected) of SD^{we} alleles. Sequences of PCR primers were obtained from the public databases (http://rgd.mcw.edu/; http://ratmap.hgc.jp). Oligonucleotides used for the amplification were sythesized in-house. PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 55/58°C for 40 s, elongation at 72°C for 40 s, 30/35 cycles. PCR products were detected either on 3% agarose gels or on 10% polyacrylamide gels under UV light upon ethidium bromide staining.

In order to identify the relative order and distances of markers and known genes on Chr 4, we also used a rat–hamster radiation hybrid (RH) panel (Research Genetics, USA). One hundred eight DNA samples from the panel were amplified with primers specific for genes and microsatellite markers. These primer sequences were selected from public databases as above, or were designed based on the recently available rat genome sequence draft (http://www.ncbi.nlm.nih.gov/genome/guide/rat/index.html). The sequence of the primers for the genes on Chr 4 is available from the authors upon request. PCR products were detected on 2% agarose gel electrophoresis. One of three symbols was assigned to each amplified sample: '1' for a positive PCR result, '0' for a negative PCR result, and '2' for an uncertain result. The results of the RH genotyping were submitted to the RH mapping server of the Rat Genome Database (RGD) (http://rgd.mcw.edu/tools/rhmapserver/rhmapserver.cgi) so that the markers and genes we analyzed were placed into the contest of framework RH maps.

Statistical and linkage analyses. For the initial genome-wide screen, the distribution of heterozygous versus homozygous genotypes among affected and unaffected animals was tested for significant deviation from randomness by using the Fischer's exact test (http://www.matforsk.no/ola/fisher.htm). All the backcross rats were phenotypically classified into either the affected (with cataracts) or the unaffected group. *P* values below 0.05 were considered indicative of linkage, and values below 0.001 were considered as proof of linkage. Interval mapping was

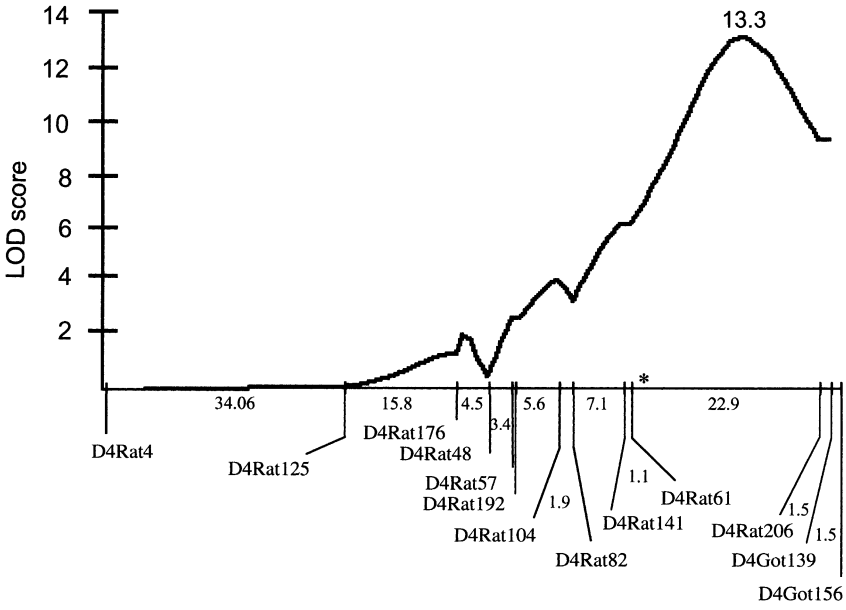

Fig. 1. LOD score graph versus map distance for genetic markers on Chr 4 obtained with the MAPMANAGER/QTX computer program. The analysis reveals a peak LOD score of 13.3 between markers *D4Rat61* and *D4Rat206*. The genetic distances between the markers were obtained from the SHRSP × BN genetic map of the RGD (http://rgd.mcw.edu/maps/). For markers *D4Rat82*, *D4Got139*, and *D4Got156*, no genetic data were available, and therefore their relative genetic distances were estimated from their physical position (Mb): their Mb position was divided by the ratio physical position/genetic position of the closest marker. The approximate position of the *Ret* gene (inferred from its physical position) is indicated with an asterisk.

performed by using the MAPMANAGER/QTX computer program (Manly et al. 2001). The physical position of the markers and genes reported in the text was obtained from the NCBI rat genome resources (http://www.ncbi.nlm.nih.gov/genome/guide/rat/index.html). The genetic position, and consequently the distance between markers, was obtained from the SHRSP × BN genetic map of the RGD (http://rgd.mcw.edu/maps/) or estimated based on their physical position (Mb) as follows: their Mb position was divided by the ratio physical position/genetic position of the closest marker. The results of radiation hybrid genotyping were analyzed with the MAPMANAGER/QT computer program (Manly et al. 1999).

Results

Linkage analysis. Forty-one (WIST/Nhg × SD^{We})F_1 × SD^{We} backcross rats were generated. The rats were examined for the presence of inborn bilateral cataracts, used as a surrogate marker for the disease (Fritz et al. 2002), and 20 had cataracts. We performed an association study analysis on those 41 animals by using the correlation between genotype and phenotype. Sixty microsatellite markers showed length variation of the PCR products between our WIST/Nhg and SD^{we} rats. *P* values for statistical significance of linkage of the informative markers were calculated by using the Fischer's exact test (data not shown). The only region of the genome showing significant linkage to the phenotype was on Chr 4, in the vicinity of marker *D4Rat61* ($P = 4.8 \times 10^{-6}$). The markers located on other chromosomes showed non-significant *P* values and therefore were not considered further. Marker *D4Rat61* was included because of its proximity to the rat homolog of the *RET* proto-oncogene, a plausible candidate gene for the disease (see below). Markers on Chr 1 in the vicinity of the rat homolog of the *MEN1* gene encoding Menin, a second possible candidate gene, were also tested but found not to be informative in our animals (data not shown). Four additional Chr 1 markers showed non-significant *P* values (data not shown).

Fine-mapping of the MENX locus. Additional microsatellite markers in the distal portion of the Chr 4 were tested on the same cohort of animals, and haplotype analysis was used to identify putative recombinant animals that could contribute to the fine-mapping of the *MENX* locus on Chr 4. Interval mapping showed the maximum LOD score of 13.3 at a position 13.4 cM distal to *D4Rat61* and 10.8 cM proximal to *D4Rat206* (Fig. 1). With the commonly applied criteria of LOD_{MAX}-1 to define the locus interval, the interval between 83.4 cM and 91.1 cM is that most likely to harbor the gene in question. In an attempt to further restrict the critical region, a panel of 10 additional microsatellite markers located in this interval were also tested (data not shown), but the SD and WIST/Nhg strains did not show length variation for any of them, except for marker *D4Rat201* (see below).

Exclusion of Ret as the putative candidate gene for MENX. The rat genome has not yet been fully annotated; therefore, we created a rat–mouse–human synteny map to gain insight into the genes that are

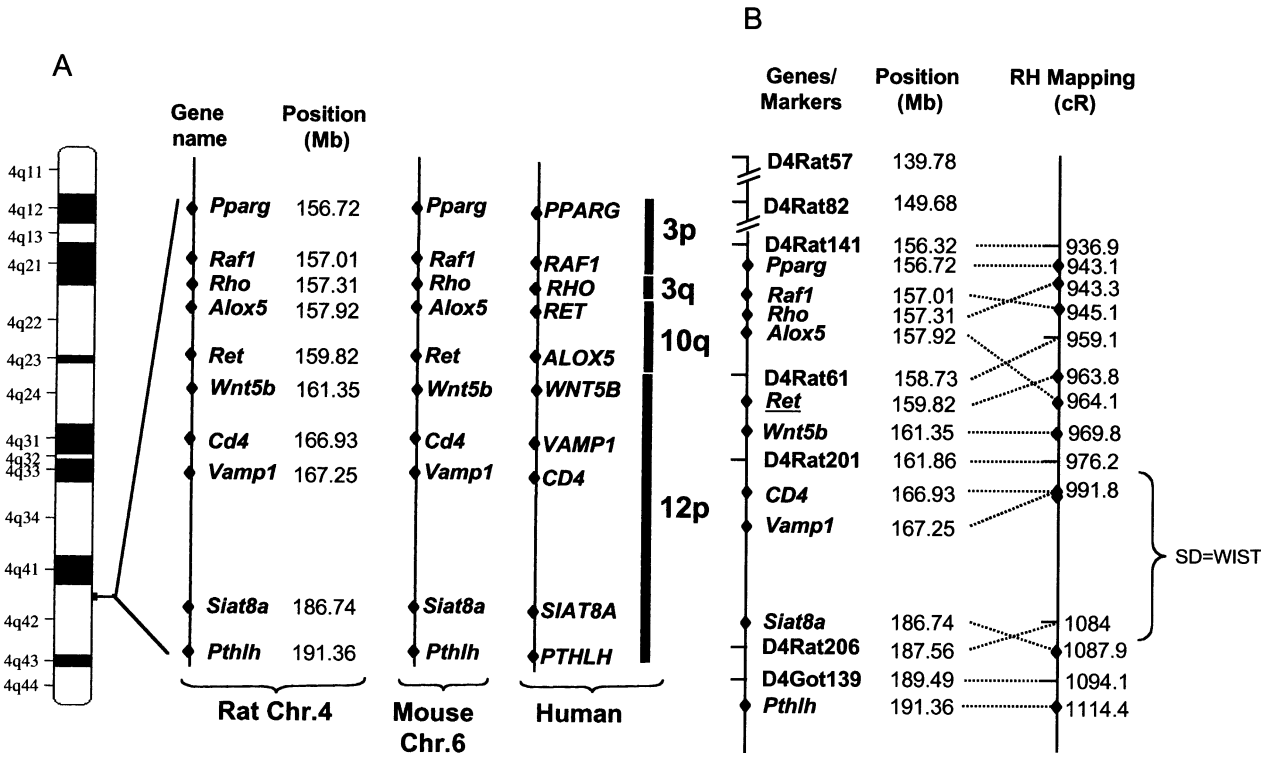

Fig. 2. Genetic, physical, RH and syntenic map of the distal part of rat Chr 4 A) Schematic representation of the rat–mouse–human synteny map. The location of the rat genes was obtained from the NCBI rat genome draft sequence. **B)** Relationship between the physical map and the RH map for microsatellite markers and genes in the region. We tested 10 additional microsatellite markers in this interval, but they were not informative in our animals. SD = Sprague Dawley. WIST = WIST/Nhg. SD = WIST, the two strains do not show length variation for 10 microsatellite markers located in this region.

located within the ~23-cM region we identified. The distal part of rat Chr 4 is syntenic to mouse Chr 6 and to portions of human Chrs 12p, 10 q, and 3 (Fig. 2A). The rat-mouse synteny map shows a highly conserved relative order of the genes analyzed. To obtain a more complete physical map of this region of Chr 4, we also used an RH panel to map markers and several known genes (Fig. 2B). Among the genes located in the distal part of rat Chr 4 is the *Ret* gene (Fig. 2A and 2B).

Because of the involvement of *RET* in the MEN2 syndrome in humans, it was important to obtain conclusive data about the possible involvement of the rat *Ret* gene in MENX, although MENX is a recessive trait and no mutations had been found by sequencing the *Ret* cDNA in SD^{we} animals (Fritz et al. 2002). Several additional microsatellite markers were identified in the close proximity of the *Ret* gene (Fig. 3A). Genotyping results obtained for marker *D4Got152*, which maps approximately 200 kb proximal to *Ret*, and marker *D4Rat201*, which maps approximately 2 Mb distal to *Ret* (Fig. 3A), are shown in Fig. 3B. Both markers are not linked to the disease locus. Figure 3C summarizes the results of haplotype analyses for several informative markers in the distal part of Chr 4, including *D4Rat61* and *D4Rat201*, proximal and distal relative to *Ret*, respectively. These data clearly exclude *Ret* as a candidate gene for MENX, since one affected animal is an inferred heterozygote at the *Ret* locus (i.e., it shows SD and WIST/Nhg alleles at both *D4Rat61* and *D4Rat201*), and four unaffected animals are inferred homozygotes (i.e., they are homozygous for the SD allele at both *D4Rat61* and *D4Rat201*) (Fig. 3C).

The latest integrated linkage map available at RATMAP (http://ratmap.gen.gu.se/gene_mapping_data/integrated_linkage_maps/) places *Ret*, together with marker *D4Rat61*, at 97 cM, and *D4Rat201* at 104 cM. Therefore, the relative order of the markers near *Ret* that we estimated from our haplotype analyses agrees with what has been reported in the database. Moreover, the estimated order of all markers used for our haplotype analyses also concords with their physical position (Fig. 3C). Notably, the genetic distances calculated based on the number of recombinant animals, among the 41 available for the study, are also in agreement with the distances reported in RGD (see Fig. 1) and

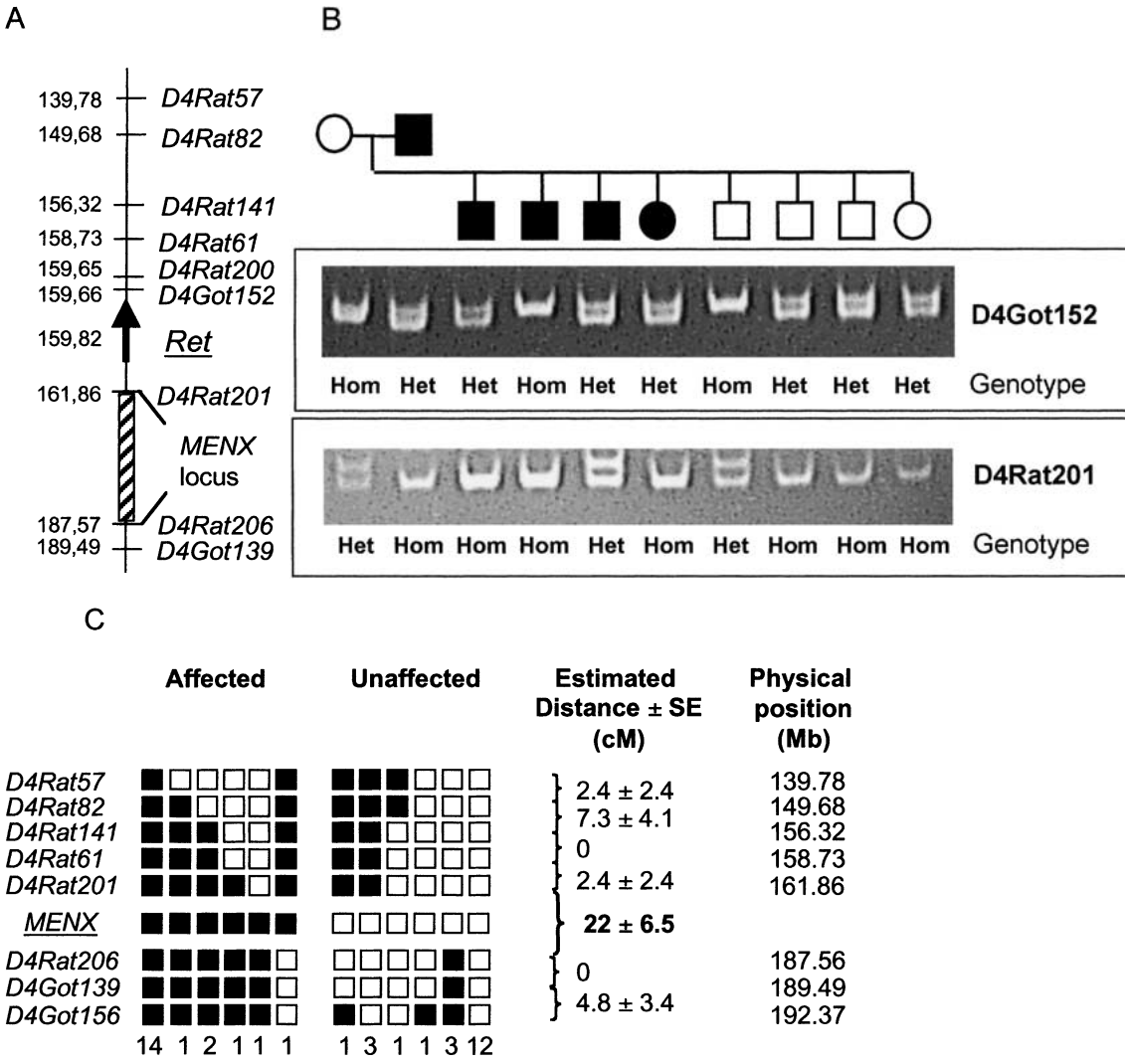

Fig. 3. Exclusion of *Ret* as the candidate gene for MENX. **A)** Informative microsatellite markers located in the vicinity of the *Ret* gene are reported together with their physical position along Chr 4. **B)** Example of genotyping and segregation analysis for markers *D4Got152* and *D4Rat201* in a subset of animals. Open square/circle: unaffected male/female; filled square/circle: affected SD^{we} male/female. Hom/Het = homozygous/heterozygous for the markers used. **C)** Haplotype analysis of affected and unaffected animals. Filled squares indicate homozygosity for a particular microsatellite marker; open squares indicate heterozygosity. The numbers below each column represent the number of animals with that particular haplotype pattern. On the right, we report the distance between markers estimated based on recombination events (± standard error), as well as the physical position of each marker based on the NCBI rat genome draft sequence. The inferred haplotype at the *MENX* locus is indicated.

RATMAP genetic maps. The only exception is marker *D4Rat206* that in RATMAP is placed distally to markers *D4Got139* and *D4Got156*. This is in contrast to both its genetic position in RGD and its physical position. Moreover, this placement would lead to two double-recombination events among the 41 animals we studied in an interval estimated by RATMAP of 20 cM. We find this an unlikely event and, therefore, prefer to rely upon our own positioning of marker *D4Rat206* (Fig. 3C).

In conclusion, haplotype analyses confirmed that the *MENX* locus maps to the ~22 cM interval between marker *D4Rat201* and marker *D4Rat206*, interval that does not include the *Ret* gene.

Discussion

We report here the mapping of the *MENX* locus to a ~22-cM region located on the distal part of Chr 4 and the exclusion of *Ret* as the candidate gene for this disease. Note that we found no *Ret* mutations in the germline of affected rats (Fritz et al. 2002). Careful analysis of the human syntenic regions using the OMIM database did not identify genes having a

specific function in development, function, or pathology of neuroendocrine organs. Ongoing efforts are focusing on the generation of additional recombinant animals that should allow us to further restrict the critical region to a size suitable to perform more systematic analyses of putative candidate genes.

Although the genetic basis of MEN syndromes has been elucidated in humans, the molecular pathogenesis of familial and sporadic tumors of MEN type is not completely understood. Genes involved in hereditary phaechromocytomas (Crossey et al. 1995; Woodward et al. 1997; Astuti et al. 2001) and paragangliomas (Baysal et al. 2000; Niemann and Muller 2000) have been recently isolated; however, they account for only a small fraction of the sporadic tumor cases (reviewed in Maher and Eng 2002). The same applies to pituitary adenomas: despite significant progress in the understanding of the molecular mechanisms underlying pituitary cell function and phenotype, pituitary tumorigenesis remains largely an enigma. Genes mutated in specific subtypes of pituitary tumors, such as the *gsp* oncogene (Landis et al. 1989; Lyons et al. 1990), putative tumor suppressor loci, and genes whose change in expression is associated with pituitary tumors, have all been reported (reviewed in Farrell and Clayton 2000). However, no major player has been identified so far, and the known genetic changes are often difficult to place into context with regard to their role in tumor formation and progression. The identification of the gene causing MENX is expected to increase our knowledge about the general mechanisms of neuroendocrine tissue tumorigenesis and to potentially provide new molecular tools for improved therapeutic approaches of MEN-type tumors.

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